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The role of Nox2 containing NADPH oxidase in hypertensive heart disease

Chaubey, Sanjay

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Cardiovascular Division
King's College London School of Medicine

THE ROLE OF NOX2 CONTAINING NADPH OXIDASE IN HYPERTENSIVE HEART DISEASE

Thesis submitted for the degree of
Doctor of Philosophy
King's College London
University of London

Sanjay Chaubey

October 2013

Dr Alison Cave
Professor Ajay M. Shah

ABSTRACT

NADPH oxidases are a family of reactive oxygen species generating enzymes which are implicated in cardiovascular pathophysiology. The Nox2-based NADPH oxidase has been reported to be involved in the development of cardiac hypertrophy and fibrosis. However the relevant cellular source(s) involved in the above effects remains unclear. The aim of this thesis was to investigate the critical cell source of the Nox2 in a model of angiotensin II (AngII) induced left ventricular hypertrophy (LVH) and fibrosis.

ROS production in resident cardiac fibroblasts was stimulated by AngII, aldosterone and TGF β to investigate cardiac fibroblasts as a crucial cell source for Nox2. Only TGF β elicited a Nox2 dependent ROS response thus placing Nox2 downstream of TGF β . As AngII stimulates cardiac fibrosis and hypertrophy via downstream TGF β , resident cardiac fibroblasts could be another critical cell source for Nox2 in the development of cardiac fibrosis and hypertrophy.

The importance of Nox2 in macrophages/monocytes (peripheral circulating cell) migration was investigated by way of Dunn Chambers. Nox2 was shown to be crucial in the chemotaxis of these cells towards CSF-1 stimulation. Also the speed and persistence of their migration was significantly reduced following the loss of Nox2.

Chimeric mice (WT mice with Nox2^{-/-} marrow and Nox2^{-/-} mice with WT marrow) were generated in-house by lethal radiation and marrow rescue with cells from mice of different genotype. Following AngII stimulation the chimeric mice demonstrated a hypertensive response. The loss of Nox2 in the circulating cells prevented the development of left ventricular hypertrophy suggesting that the peripheral circulating cells were a critical cell source for Nox2 in the development of cardiac hypertrophy. The loss of Nox2 in the resident cardiac cells prevented the development of cardiac fibrosis thus suggesting that the resident cardiac cells were the critical cell source for Nox2 in the development of cardiac fibrosis.

These results indicate that Nox2 has a cell specific role in the development of cardiac fibrosis and hypertrophy following AngII stimulation.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ACE	Angiotensin converting enzyme
Akt	Serine / threonine protein kinase B
Ang II	Angiotensin II
AT-1	Angiotensin II subtype 1 receptor
AU	Arbitrary units
BH ₄	(6R)-5,6,7,8-tetrahydrobiopterin
BMM	Bone marrow derived macrophages
BW	Body weight
CABG	Coronary artery bypass grafts
CAD	Coronary artery disease
CCR1	Chemokine receptor 1
CD62L	L selectin
COX	Cyclooxygenase
CSF-1	Colony-Stimulating Factor
CX3C	Neurotactin or fractalkine
CX3CR1	CX3C receptor
DBP	Diastolic blood pressure
DCC	Dunn chemotaxis chamber
DDR2	Discoidin domain receptor
DHE	Dihydroethidium
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DPI	Diphenylene iodonium
EC	Endothelial cells
ECM	Extracellular matrix
EMT	Epithelial mesenchymal transition
EndoMT	Endothelial mesenchymal transition
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FAD	Flavin adenine dinucleotide
FITC	Fluorescein isothiocyanate
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor

GPCR	G protein-coupled receptors
GSH	Glutathione
GSSG	Glutathione disulphide
GTP	Guanosine triphosphate
HMG-CoA	Three-hydroxy-3-methylglutaryl coenzyme A
iNOS	Inducible nitric oxide synthase
JNK	c-Jun-N-terminal kinase
ko	Knockout
LEC	Lucigenin enhanced chemiluminescence
L-NAME	N ^G -nitro-L-arginine methyl ester
LOX	Lipoxygenase
LTB ₄	Leukotriene B ₄
LV	Left ventricle/ left ventricular
Ly6C	epitope of granulocyte receptor
MAO	Monoamine oxidase
MAP	Mean arterial pressure
MAPK	Mitogen activated protein kinases
MCP-1	Monocyte chemotactic protein-1/CCL2
MI	Myocardial infarction
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
mRNA	messenger RNA
NADPH	Nicotinamide-adenine-dinucleotide phosphate
NF κ b	Nuclear factor kappa-light-chain-enhancer of activated B cells
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO	Nitric oxide
NOS	Nitric oxide synthase
O ₂ ^{•-}	Superoxide anion radical
OH [•]	Hydroxyl radical
ONOO ⁻	Peroxynitrite
PCF	Primary cardiac fibroblast
PDE-5	Phosphodiesterase-5
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PGI ₂	Prostacyclin
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
RAAS	Renin angiotensin aldosterone system
RAS	Renin angiotensin system

RBC	Red blood cell
RNA	Ribonucleic acid
ROCK	Rho-kinase
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SMC	Smooth muscle cell
SOD	Superoxide dismutase
TGF β	Transforming growth factor
TIMP	Tissue inhibitors of metalloproteinases
TRX	Thioredoxin
TTFA	Thenoyltrifluoroacetone
VH	Ventricular hypertrophy
vWF	von Willebrand Factor
WT	Wild type
XD	Xanthine dehydrogenase
XO	Xanthine oxidase

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DECLARATION

I declare that I am the sole author of this thesis and that it contains my own work except where otherwise acknowledged.

1. INTRODUCTION

1.1. HYPERTENSION

Hypertension is a common medical condition in which a person's blood pressure is chronically elevated and can be classified as either essential (primary) or secondary. Essential hypertension accounts for 90-95% of the cases of hypertension and is defined as hypertension in which no cause underlies the raised blood pressure (Carretero and Oparil, 2000). It is estimated that nearly one billion people are affected by hypertension worldwide, and this figure is predicted to increase to 1.5 billion by 2025 (Kearney et al., 2005). Secondary hypertension indicates that the hypertension is a result of (ie secondary to) another condition such as kidney disease, tumors or adrenal disease.

The British Hypertension Society recommends that drug therapy be started in patients with a sustained systolic blood pressures of greater than or equal to 160 mmHg or sustained diastolic blood pressures of greater than or equal to 100 mmHg despite non-pharmacological measures (Williams et al., 2004). Treatment is recommended as persistent hypertension is a well established risk factor for developing end organ damage typically in the kidneys (Wang et al., 2009a), eyes (Wong and Mitchell, 2007), arteries (Insull, Jr., 2009), brain (Papadopoulos and Votteas, 2006) and the heart (Murphy et al., 2009; Papadopoulos and Papademetriou, 2010).

Even though no direct cause is known for essential hypertension there are many risk factors such as sedentary lifestyle (Kyrou et al., 2006), obesity (Segura and Ruilope, 2007; Wofford and Hall, 2004), alcohol (Djousse and

Mukamal, 2009), age (Kosugi et al., 2009) and genetic mutations (Dickson and Sigmund, 2006; Gong and Hubner, 2006; Kotchen et al., 2000). The mechanism underlying essential hypertension is not well understood but what is known is that with time there is an increase in the total peripheral resistance which results in high blood pressure. One of the proposed theories for this increased total peripheral resistance is an overactive renin-angiotensin system (Figure 1.1) which leads to greater formation of AngiotensinII (AngII) resulting in vasoconstriction of arteries and retention of sodium and water in the kidneys, the net result of which is to increase blood pressure (Sowers et al., 2009). This is supported by genetic linkage between the angiotensinogen gene and hypertension in many studies in which there is the presence of increased levels of plasma angiotensinogen (AngII precursor) in hypertensive subjects (Naber and Siffert, 2004).

1.2. RAAS

The renin-angiotensin system (RAS) or the renin-angiotensin-aldosterone system (RAAS) (Figure 1.1) is a hormone system that regulates blood pressure and water (fluid) balance. The classical view is that the RAS cascade exists in the circulation. The classical circulatory RAS cascade is initiated by production and secretion of the enzyme renin from the kidney's juxtaglomerular apparatus in response to stimuli such as a reduction in circulating blood volume. Renin is considered a key enzyme of RAS due to the rate limiting nature of its hydrolytic activity on angiotensinogen. Recently there has also been the discovery of a renin/prorenin receptor the function of which is

to be fully elucidated (Nguyen et al., 2002). Angiotensinogen is released into the circulation, predominately from the liver. AngII is formed by the enzymatic cleavage of angiotensinogen by renin to form the inactive peptide, Angiotensin I. This in turn is converted to AngII by angiotensin converting enzyme (ACE). This enzyme is highly expressed in the endothelial cell membrane of the pulmonary circulation (Weber, 2001; Zaman et al., 2002). Another action of ACE outside the RAS is the inactivation of the vasodilator bradykinin further strengthening the role of ACE and therefore the RAS as being pro-hypertensive.

However recent discoveries have contributed to changing our understanding of RAS from the classical linear cascade to a cascade with multiple mediators, receptors and enzymes (Chappell, 2007; Santos and Ferreira, 2007; Simoes e Silva AC et al., 2006). It is now appreciated that the RAS also exists in distinct organs such as the heart which has been termed the local RAS (Miyazaki and Takai, 2006; Paul et al., 2006). The local RAS has also been found in blood vessels, kidney, adrenal glands, pancreas adipose tissue, nervous system and reproductive system (Lavoie and Sigmund, 2003; Nielsen et al., 2000; Sernia, 2001; Spat and Hunyady, 2004). The local RAS appears to be regulated independently of the circulatory RAS but can interact with it. The effect of the local RAS can occur on the cell itself (autocrine), on adjacent cells (paracrine) or through the bloodstream to a specific organ or tissue (endocrine) (Miyazaki and Takai, 2006; Paul et al., 2006). However the main importance of local RAS is linked to local regulatory mechanisms that control many homeostatic pathways (Miyazaki and Takai, 2006; Paul et al., 2006).

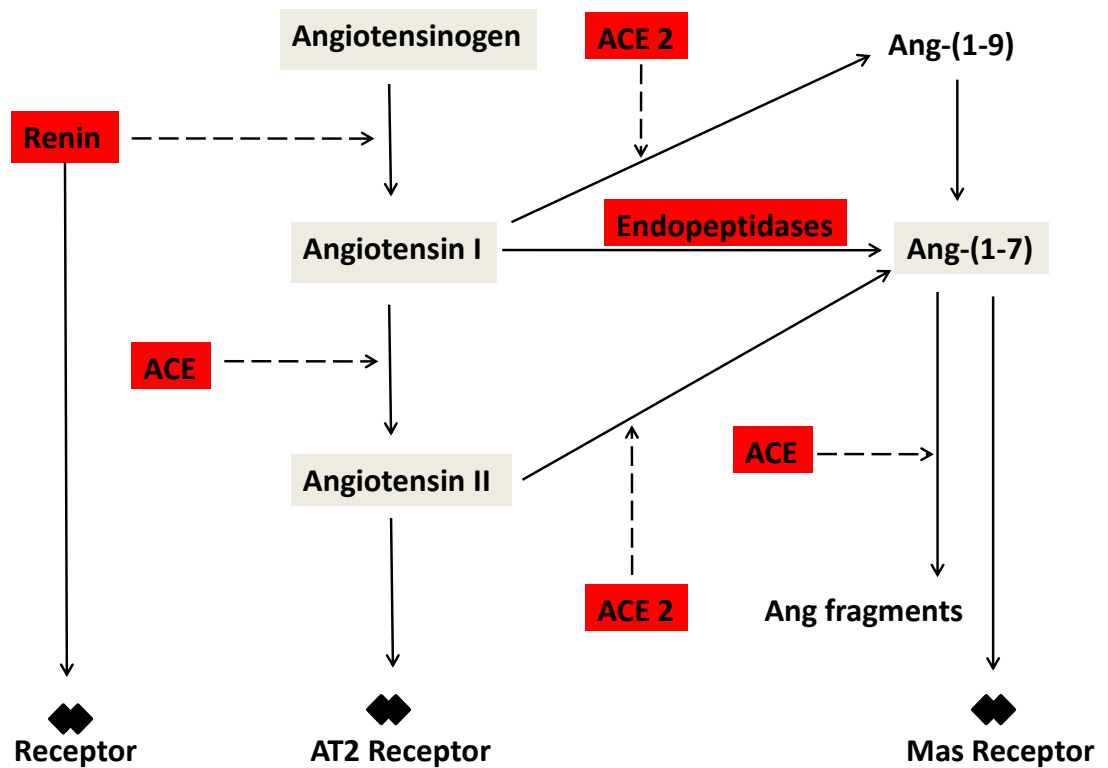


Figure 1-1 Renin angiotensin system

Renin can act via its own newly discovered receptor the function of which is not fully known. Its established role is in converting angiotensinogen to the inactive Ang I. Angiotensin converting enzyme (ACE) converts Ang I to Ang II which via its AT1 receptor mediates the hypertensive roles of Ang II. ACE2 removes Ang II by reducing the availability of Ang I and by converting it to Ang-(1-7). Ang-(1-7) actions via its Mas receptor are counter regulatory to those mediated by the Ang II-AT1 receptor.

Recently, an ACE homologue, ACE2 was discovered (Figure 1.1), which is expressed in the heart (Gembardt et al., 2005). In contrast to ACE, ACE2 does not metabolise bradykinins. The catalytic efficiency of ACE2 against AngII is 400 fold higher than for AngI. This enzyme can degrade AngII to angiotensin fragments called Ang(1-7), and also cleaves AngI into Ang(1-9) (Hamming et al., 2007). The identification of Mas as a G-protein coupled receptor of Ang-(1-7) established this heptapeptide as a biologically active member of the RAS cascade.(Figure 1.1) Most evidence supports a counter regulatory role for Ang-(1-7) by opposing many AngII-AT₁ receptor mediated actions (Chappell, 2007; Santos and Ferreira, 2007; Simoes e Silva AC et al., 2006). Indeed in some tissues ACE2 is the main isoform for Ang-(1-7) generation. Therefore, ACE2 acts as a clearance mechanism directly removing AngII and working indirectly on AngI, therefore reducing the ability to produce AngII. The RAS can be seen as a dual function system in which the vasoconstrictor or vasodilator actions are primarily driven by the ACE/ACE2 balance. Furthermore, the principle enzymes in the classical cascade, renin and ACE, are not the only enzymes which can conduct the reaction. Production of AngII from AngI can be driven by a protease chymase instead of ACE (Kinoshita et al., 1991), whereas renin can be replaced by cathepsin D (Genest et al., 1983). In local RAS, chymase may be the dominant enzyme producing AngII, therefore treatment with ACE inhibitors will not prevent AngII being produced locally by chymase (Wolny et al., 1997). Basal expression of the RAS components is low in the heart, however, during pathological conditions, the local RAS in the heart becomes activated (Kumar et al., 2009). The significance of local versus systemic RAS system is yet to be fully

defined however there is evidence to suggest that in many disease settings local RAS may play a role (Koka et al., 2006; Kumar et al., 2008; Li et al., 2004; Singh et al., 2008).

AngII acts via its receptor named AngII receptor of which there are two subtypes namely AT₁ and AT₂. The G-protein coupled AT₁ receptor mediates the known physiological and pathological actions of AngII and undergoes rapid desensitisation and internalisation after agonist stimulation. It is the AT₁ receptor which mediates the hypertensive effects of Ang II (Touyz and Schiffrin, 2000). Throughout the body, AngII is a potent vasoconstrictor and this results in an increase in the total peripheral resistance. Its action in the adrenal gland is to stimulate the release of aldosterone which acts on the kidneys to again increase fluid reabsorption. Physiologically the net result of this increased fluid retention and increase in peripheral resistance is to increase the blood pressure. The AT₂ receptor acts through G_i and tyrosine phosphatases to exert predominantly inhibitory actions on cellular responses mediated by the AT₁ receptor. For example, both receptors play a role in regulating vascular smooth muscle cell function. While AT₁ receptor is associated with vasoconstriction AT₂ receptors generally have the opposite effect of vasodilatation (Touyz and Schiffrin, 2000).

Ang-(1-7) is formed from AngII by the action of endopeptidases or by ACE2. Alternatively it can be formed from AngI by the action of ACE2 forming Ang-(1-9) in the process. ACE inhibitors elevate plasma Ang-(1-7) concentration by both increasing AngI, the substrate for Ang-(1-7) as well as preventing Ang-(1-7) degradation. The recent identification of ACE2 which forms Ang-(1-7), the

Mas receptor as an Ang-1(1-7) receptor and its counter regulatory role of AngII induced effects has given it more biological significance (Jin et al., 2012). In this regard, at least in part, some of the beneficial effects of ACE inhibitors in the heart and kidney may be attributed to Ang-(1-7) (Santos and Ferreira, 2007; Simoes e Silva AC et al., 2006). Thus the RAS may act through two opposite arms. The major one responsible for systemic actions is constituted by ACE-AngII-AT₁ receptor axis and the counter regulatory arm formed by ACE2-Ang-(1-7)-Mas axis (Santos and Ferreira, 2007; Simoes e Silva AC et al., 2006).

1.3. EFFECT OF HYPERTENSION ON THE HEART

1.3.1 STRUCTURE OF THE EXTRACELLULAR MATRIX OF THE HEART

The normal myocardium is composed of a variety of cells. Cardiac myocytes occupy a major portion of tissue space however represent only one third of all cells. The remaining two thirds and include endothelial and vascular smooth muscle cells of the intramural coronary vasculature and fibroblasts located in both interstitial and perivascular spaces. The cells are tethered within an extracellular scaffolding of fibrillar collagen termed the extra cellular matrix (ECM).

The extra cellular matrix (ECM) is made up of a complex lattice of structural proteins e.g. collagen and elastin, as well as adhesive proteins e.g. laminin and fibronectin, which surround the cardiomyocytes. Collagens are the major constituent of the ECM, with the fibrillar collagen types I and III being the most abundant in normal hearts, and their diverse structural properties play an important part in the structure and function of the heart (Graham et al., 2008).

Collagen I has a high tensile strength, which therefore, determines cardiac stiffness, while the distensible property of collagen type III maintains cardiac structure (Weber, 1989). Associated with the collagens are elastic fibers, which are responsible for passive recoil; these fibers are made from an elastin core covered in fibrillin microfibrils. Adhesive proteins including fibronectin, laminin and collagen typeIV bind ECM structural proteins to the cardiomyocytes providing structural support.

The ECM is the structural component that maintains the alignment of the myocytes preventing slippage during contraction. As a passive functional component it is the main determinant of diastolic stiffness. However the ECM is not an inert component and plays an essential role in the transmembrane signaling via integrins leading to MAP kinase activation and cytoskeletal rearrangement of the cells tethered to the ECM.

1.3.2 HYPERTROPHY

An increase in peripheral resistance imposes a greater workload on the heart which the heart compensates by developing a muscular hypertrophy. Ventricular hypertrophy (VH), defined as an increase in overall ventricular muscle mass, can occur naturally as a reaction to aerobic and anerobic exercise. In aerobic training, the stress imposed on the heart is volume overload as blood return to the heart increases via increased contraction of the skeletal muscle (skeletal-muscle pump). As a result aerobic training results in the heart being able to pump a larger volume of blood through an increase in the size of the ventricles (Pluim et al., 2000). The ventricular cardiomyocytes respond by

adding new sarcomeres in-series with existing sarcomeres (i.e the sarcomeres lengthen rather than thicken). This results in ventricular dilatation while maintaining normal sarcomere length. The wall thickness normally increases in proportion to the increase in chamber radius. This type of hypertrophy is termed eccentric hypertrophy.

Anaerobic training results in the thickening of the myocardial wall in order to push blood through arteries compressed by muscular contraction. Therefore the stress imposed on the heart in anaerobic training is pressure overload. Wall thickness is increased as new sarcomeres are added in-parallel to existing sarcomeres in the ventricular cardiomyocytes. However the chamber radius may not change. This type of hypertrophy is termed concentric hypertrophy (Pluim et al., 2000). The ventricles are capable of generating greater forces and higher pressures, while the increased wall thickness maintains normal wall stress.

Pathological hypertrophy can be caused by an increase in the afterload as a result of, for example aortic stenosis and hypertension. The stress imposed on the heart is chronic pressure overload and the left ventricle responds by developing concentric hypertrophy. Pathological cardiac hypertrophy is associated with changes in gene expression in the cardiomyocytes and re-expression of “foetal” genes such as atrial natriuretic factor (ANF), β -myosin heavy chain (β -MHC) and skeletal α -actin is seen (Sadoshima and Izumo, 1997). Such cardiac hypertrophy can cause a rapid change in the isoform of the myofibril which results in a reduction in the rate of shortening. This change is thought to be an adaptation of the heart to conserve energy however, this may

also lead to a reduced force of contraction and contribute to the maladaptation (Gupta, 2007).

The VH itself is not a disease but serves as an independent risk factor for increasing the future incidence of myocardial infarction, stroke, sudden cardiac death and heart failure (Boyd et al., 2012; Cicala et al., 2007; Gerds et al., 2012). The hypertrophied ventricle is stiff and less compliant and is therefore unable to relax as effectively and this reduces the diastolic filling of the heart. At a cellular level the cardiac myocyte hypertrophy may or may not be accompanied by the growth of noncardiomyocyte cells. The cardiac tissue homogeneity is preserved with the cardiac myocyte growth that appears in response to ventricular pressure or volume overload. Such adaptive hypertrophy is observed in athletes (Di, V et al., 1997), patients with chronic anemia (Bartosova et al., 1969), arteriovenous fistula (Michel et al., 1986), atrial septal defect, or hyperthyroidism (Bonnin et al., 1983). However this is not associated with VH following activation of RAS.

Cardiac hypertrophy seen in hypertensive heart disease is associated with activation of the RAAS (Kim and Iwao, 2000; Mehta and Griendling, 2007; Weber et al., 1999). In time fibrosis develops in the hypertrophied heart impairing the diastolic compliance further resulting in what is termed as diastolic dysfunction. The hypertrophy also negatively influences the energetics of the cardiomyocytes resulting in their dysfunction (Sambandam et al., 2002). The impairment of cardiomyocyte function results in the impairment in the cardiac contraction of the heart, termed systolic dysfunction. The heart with both diastolic and systolic dysfunction is now said to be in total heart failure.

1.3.3 CARDIAC FIBROSIS

1.3.3.1 The role of RAS

Fibrosis is multifactorial and can result from a variety of causes including myocardial ischaemia, senescence, diabetes and hormones such as AngII amongst other causes. It is common to identify two different types of fibrosis, namely, reparative and reactive fibrosis. Reparative fibrosis occurs as a reaction to a loss of myocardial tissue due to necrosis or apoptosis, and is mainly interstitial. In contrast, reactive fibrosis is observed in the absence of cell loss as a reaction to inflammation and is primarily perivascular (Swynghedauw, 1999).

Such adverse remodelling of the heart's non-muscular compartment in hypertension is not necessarily due to the mechanical pressure overload. In models of infra-renal aortic banding with normal circulating levels of AngII and aldosterone, there is no evidence of increased cardiac fibrosis despite the presence of significant hypertrophy (Brilla et al., 1990). Similarly chronic elevation of noradrenaline levels is also not associated with increased cardiac fibrosis despite an elevation in blood pressure (Patel et al., 1989). Furthermore in human hypertension, fibrosis has been found in both the left and right ventricles in biopsies of myocardial tissue, despite the right heart pumping blood against a reduced afterload, suggesting that humoral factors are more important than the mechanical loading in the bringing about the fibrotic response (Amanuma et al., 1994). Raised levels of AngII are thought to play a key role in triggering the inflammatory response resulting in fibrosis. In support of this many studies have shown that the blockade of AngII or its action results in a reduction in fibrosis. For example Tokuda et al (Tokuda et al., 2004)

demonstrated that a sub-depressor dose of candesartan (an angiotensin II receptor blocker) in mice who had had supra-renal aortic banding markedly ameliorated perivascular fibrosis.

One of the ways AngII is thought to initiate fibrosis is by up-regulating the expression of macrophage chemoattractant protein-1 (MCP-1), which is a major regulator of macrophage recruitment into inflammatory tissues. It has been observed that pressure overloaded hearts show an increased expression of MCP-1 which was accompanied by macrophage infiltration in the perivascular space of both small and large intramyocardial arteries (Capers et al., 1997; Tokuda et al., 2004). Administration of sub-depressor dose of candesartan significantly suppressed MCP-1 induction and macrophage accumulation suggesting that AngII induces reactive fibrosis through mechanisms that are independent of the pressor effect (Tokuda et al., 2004).

1.3.3.2 Role of macrophages

Macrophage infiltration is a key early event in the development of reactive myocardial fibrosis (Kuwahara et al., 2003) and Tokuda et al found the accumulation of macrophages to be due to MCP-1 (Tokuda et al., 2004). The importance of macrophage infiltration for the development of fibrosis is demonstrated by the blockade of MCP-1 and ICAM-1 early in this process. The blockade of either prevented the onset of myocardial fibrosis suggesting their coordinated role towards macrophage recruitment in pressure overloaded myocardium (Kuwahara et al., 2003; Kuwahara et al., 2004).

1.3.3.3 Origin of fibroblast/myofibroblasts

The origins of activated fibroblasts in the fibrotic process are diverse. Firstly the proliferation and activation of tissue resident fibroblasts in response to profibrotic cues originating from infiltrating inflammatory cells can lead to quiescent fibroblasts expressing a myofibroblasts phenotype (Postlethwaite et al., 2004). A second source is the recruitment of fibroblast precursor cells from the bone marrow. These cells are called fibrocytes and they possess the ability to migrate from the bloodstream in response to specific chemokine gradients and localize in tissue undergoing pathological fibrogenesis (Bellini and Mattoli, 2007; Herzog and Bucala, 2010). A third source of myofibroblasts is by way of transformation of epithelial cells to myofibroblasts, a process known as epithelial mesenchymal transition (EMT). Such EMT has been shown to occur in the course of renal fibrosis, pulmonary fibrosis and liver fibrosis (Rastaldi, 2006; Willis et al., 2006; Willis and Borok, 2007; Zeisberg and Kalluri, 2008). More recently endothelial to mesenchymal transition (EndoMT) has been shown to contribute in the development of fibrosis (Zeisberg et al., 2007b). This is a complex biological process in which endothelial cells lose their specific endothelial cell markers such as vascular endothelial cadherin (VE cadherin) and initiate expression of mesenchymal cell products such as α smooth muscle actin and vimentin. These cells can also become motile and are capable of migrating into the surrounding tissue. Goumans et al (Goumans et al., 2008) pointed out that in cardiac fibrosis EndoMT may be the most important contributor towards the generation of fibrotic tissue.

1.3.3.4 Role of fibroblasts

The infiltrating macrophages in the perivascular space co-localise with fibroblasts. The macrophages are known to secrete profibrotic cytokines such as TGF- β_1 . These in turn can cause proliferation of the fibroblasts and their differentiation to myofibroblasts, which synthesise collagen (Kai et al., 2005). Kuwahara et showed that the perivascular accumulation of macrophages preceded TGF- β_1 expression and fibroblast proliferation (Kuwahara et al., 2004). Activated myofibroblasts can themselves express TGF- β_1 and other profibrotic substances which self-amplifies the ongoing fibrotic process (Desmouliere et al., 1993). Therefore macrophage infiltration is crucial as an upstream event of myocardial fibrosis with further downstream macrophage TGF- β_1 expression and fibroblast activation.

1.3.3.5 Disruption of the equilibrium of collagen synthesis

The collagen synthesis and degradation in the heart is kept in equilibrium. This process is maintained by a variety of hormones and cytokines of which matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are important (Graham et al., 2008). The effector hormones of the renin-angiotensin system, inflammatory cytokines (eg TGF- β_1), growth factors (eg insulin-like growth factor) and degradation fragments from increased MMP activity, have been shown to have potent profibrotic effects. By contrast, bradykinin, nitric oxide, natriuretic peptide, and glucocorticoids have been shown to promote collagenolysis. An excess of stimulators, due either to absolute stimulator overproduction or to their relative overabundance because

of a deficit in inhibitor formation, can promote fibrosis. In the setting of hypertensive heart disease (HHD), this equilibrium is disrupted, with activation of the RAS system and overproduction of AngII leading to fibrosis.

As fibrosis progresses the existing collagenous matrix increases in dimension resulting in interstitial cardiac fibrosis. There is accumulation of thick collagen fibres around small intramyocardial coronary arteries resulting in perivascular fibrosis (Jalil et al., 1988). These fibres then radiate into the intermuscular spaces and may merge with co-existing interstitial cardiac fibrosis. Later, areas of cell necrosis are observed and lead to microscopic scar formation (Weber et al., 1989).

A key mediator of the pro-fibrotic effect of AngII is oxidative stress. Oxidative stress is also implicated in the downstream signalling pathways of the RAAS in many pathological settings.

1.4 OXIDATIVE STRESS AND REDOX SIGNALING

Oxidative stress can be defined as the oxidative cellular damage resulting from the a pathological imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses (Griendling and FitzGerald, 2003a; Mehta and Griendling, 2007; Paravicini and Touyz, 2008; Weber et al., 1999). Thus it can result from either excess production of ROS or alternatively deficiencies in antioxidant systems.

Reactive oxygen species (ROS) are oxygen based molecules which have a high chemical reactivity. Examples of such ROS include superoxide (O_2^-),

hydrogenperoxide (H_2O_2), peroxynitrite (ONOO^-) and hydroxyl radicals (OH^-) which all aerobic organisms generate. Traditionally they have been considered to be deleterious since they can induce free-radical oxidative damage to DNA and other macromolecules. However under normal physiological conditions ROS is produced in quantities required for signaling. As part of this signaling the ROS is converted by existing antioxidant systems thus preventing the indiscriminate production of ROS. Clearly, perturbation of this system due to increased ROS production or a decrease in the antioxidant system can result in an increased oxidative state within the cells and tissues, termed oxidative stress.

Recently it has been recognised that oxidative stress can play a role in the pathophysiology of many diseases such as diabetes, hypertension, atherosclerosis, cardiac hypertrophy, heart failure and cardiac fibrosis via modulation of a number of diverse intracellular signalling pathways such as MAPK, Akt, JNK, and transcription factors such as NF κ B (Ejiri et al., 2003; Fukui et al., 1997; Griendling and Alexander, 1997; Griendling and FitzGerald, 2003a; Griendling and FitzGerald, 2003b; Grieve et al., 2004; Jung et al., 2004; Paravicini and Touyz, 2006; Sawyer et al., 2002). The modulatory effect of ROS in the pathophysiological signalling (Figure 1.2) of such diseases is termed 'redox signalling' (Jung et al., 2004; Paravicini and Touyz, 2006; Suzuki and Griendling, 2003).

The signalling properties of ROS is achieved by mechanisms such as the reversible oxidation of redox sensitive target proteins (e.g. tyrosine phosphatase) or the formation of cysteine disulfide bonds within signalling

proteins. Alternatively interaction of O_2^- with the signalling free radical nitric oxide (NO) reduces NO availability, thus modulating NO dependant responses and producing the toxic free radical peroxynitrate in the process. When O_2^- is produced it is rapidly dismutated to H_2O_2 , by the superoxide dismutase (SOD) enzyme. H_2O_2 can diffuse across cell membranes and may therefore be responsible for redox signalling further away from the initial cellular source (Rao et al., 1993a; Rao et al., 1993b; Zafari et al., 1998). Extracellular ROS production by macrophages can activate MMPs which can degrade the collagen based ECM (Galis et al., 1995; Rajagopalan et al., 1996b).

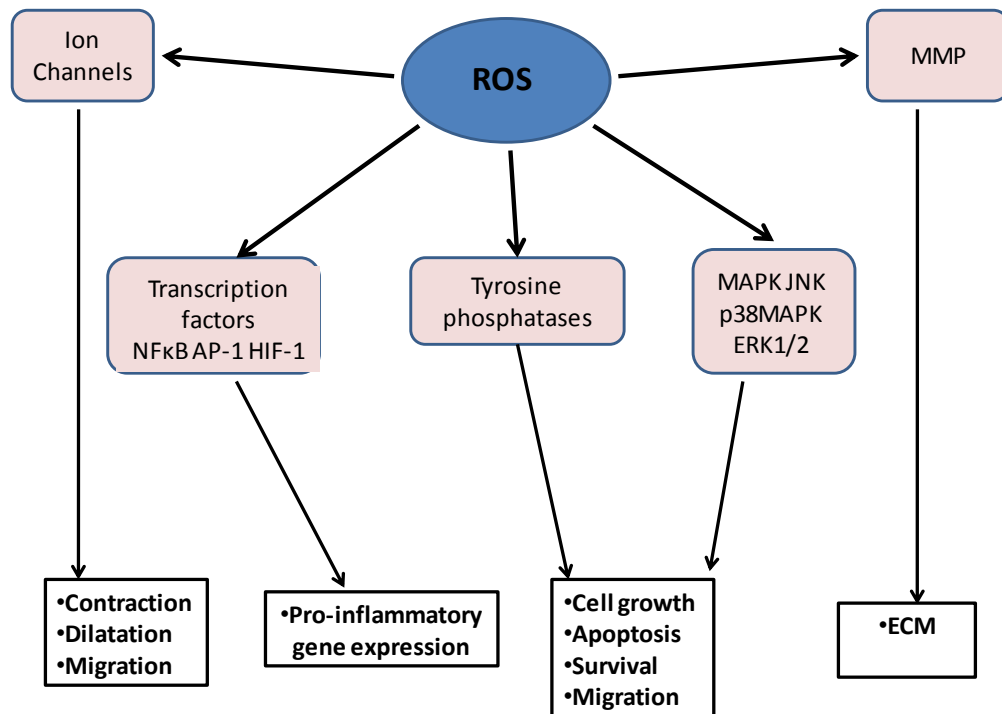


Figure 1-2 Redox dependant signalling pathways

Intracellular ROS modify the activity of tyrosine kinase as well as MAPK. ROS may inhibit protein tyrosine phosphatase activity and influence gene and protein expression by activating transcription factors. ROS can also stimulate plasma membrane Ca^{2+} and K^{+} channels leading to changes in cation concentration. Activation of these redox sensitive pathways results in numerous cellular responses.

1.5 SOURCES OF ROS

ROS are produced from a number of sources such as the mitochondria, nitric oxide synthase (NOS) and xanthine dehydrogenase. In addition, the enzyme complex NADPH oxidase has been demonstrated to be an important source of ROS.

1.5.1 *DYSFUNCTIONAL NITRIC OXIDE SYNTHASE*

Nitric oxide (NO) is an important signalling molecule with particular relevance for normal endothelial function. There are 3 isoforms of NOS all of which may be expressed in the heart. These are endothelial NOS (eNOS) and neuronal NOS (nNOS), which are constitutively expressed, and inducible NOS (iNOS).

NOS exists as a dimer with two subunits connected via a central calcium/calmodulin-binding region. NO and L-citrulline are generated by NOS through catalysing the oxidation of L-arginine. This requires the transfer of electrons from NADPH and NOS cofactor tetrahydrobiopterin (H_4B), with water formed as a by-product (Sears et al., 2004). However in situations where either L-arginine or H_4B are limiting, NOS can generate superoxide radicals. These findings have led to the concept of “NOS uncoupling”, where the activity of the enzyme for NO production is decreased in association with an increase in NOS-dependant superoxide radical formation (Heitzer et al., 2000; Katusic, 2001). Moreover H_4B is readily oxidised so that any setting where there is a significant degree of oxidative stress can result in the situation where NOS is uncoupled secondary to H_4B depletion (Laursen et al., 2001).

1.5.2 MITOCHONDRIAL ELECTRON TRANSPORT CHAIN

In the Krebs's cycle, reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH) are generated by the oxidation of nutrients such as glucose. Electrons derived from NADH and FADH are then transferred along a series of electron transport carriers - Complex I (NADH dehydrogenase), Complex II (succinate dehydrogenase), Complex III (cytochrome bc₁) and Complex IV (cytochrome oxidase) which are located on the inner mitochondrial membrane. This results in the transfer of electrons to molecular oxygen to form water. The proton gradient generated by this process is used for the synthesis of adenosine triphosphate (ATP). During this process a variety of oxygen intermediates are formed such as hydrogen peroxide and superoxide (Sena and Chandel, 2012). Monoamine oxidase A and B are also found anchored to the outer mitochondrial membrane where they have an important role in the oxidative deamination of amines such as serotonin, dopamine and epinephrine using oxygen molecule as the electron acceptor (Edmondson et al., 2009). Importantly high levels of antioxidant enzymes such as MnSOD and catalase are present which limit the total quantity of these reactive intermediates. Nevertheless it is estimated that under physiological conditions superoxide may leak from the respiratory chain. This amount may increase significantly in pathological settings (Sena and Chandel, 2012).

1.5.3 XANTHINE OXIDOREDUCTASE

Xanthine oxidoreductase is a ubiquitous metalloflavoprotein that appears in two interconvertible yet functionally distinct forms. These are xanthine dehydrogenase (XD), which is constitutively expressed *in vivo* and xanthine oxidase (XO) (Meneshian and Bulkley, 2002). XO is generated by the posttranslational modification of XD, either through the reversible oxidation of sulfhydryl residues on XD or the irreversible proteolytic cleavage of a segment of XD, which occurs at low oxygen tension and in the presence of several proinflammatory mediators. Functionally, both XD and XO catalyse the oxidation of purines to urate. However, whereas XD requires NAD⁺ as an electron acceptor for these redox reactions, thereby generating the stable product NADH, XO is unable to use NAD⁺ as an electron acceptor, requiring instead the reduction of molecular oxygen for this purine oxidation and generating the highly reactive superoxide free radicals.

Xanthine oxidase-derived ROS are known to be important in ischemia-reperfusion injury and play a role in endothelial dysfunction (Brown et al., 1988; Spiekermann et al., 2003). During ischemia and exposure of XD to low oxygen tension, irreversible proteolytic cleavage might convert XD to XO (Battelli et al., 1992), thereby priming the system for the triggering of microvascular inflammation by the generation of ROS upon the subsequent delivery of oxygen at reperfusion. The ROS thereby generated from XO could therefore trigger the accumulation, activation and diapedesis of neutrophils, with consequent neutrophil-mediated microvascular inflammatory injury, leading ultimately to parenchymal organ dysfunction.

1.5.4 LIPOXYGENASE/CYCLO-OXYGENASE

Arachidonic acid (AA) is released from glycerophospholipids in the nuclear envelope and from the plasma membrane via the activity of cytosolic phospholipase A₂ and is subsequently metabolised by cyclooxygenase (COX) and lipoxygenase (LOX) to generate a variety of bioactive eicosanoids, including prostaglandins, thromboxanes and leukotrienes (Funk, 2001; Samuelsson et al., 1987). ROS can be generated as a byproduct during the oxidation processes of AA by COX and LOX (Edderkaoui et al., 2005).

1.6 ANTIOXIDANT SYSTEM

The body has several potential endogenous antioxidant defences to protect against the overproduction of ROS. The defence mechanisms include enzymatic defences such as the superoxide dismutase (SOD) family, catalase and the glutathione peroxidase enzyme system (Marczin et al., 2003). Non-enzymatic systems include ascorbic acid (vitamin C), α -tocopherol (vitamin E), glutathione, β -carotene and vitamin A (Gotto, 2003). Vitamins are major antioxidant in humans. The vitamins act as a potent free radical scavenger that can react with oxygen, superoxide and the hydroxyl radical (Abudu et al., 2004).

1.6.1 SUPEROXIDE DISMUTASE

Superoxide radical spontaneously dismutates to O₂ and hydrogen peroxide (H₂O₂) quite rapidly. However the dismutation rate is second order with respect to initial superoxide concentration. Thus, the half-life of superoxide, although very short at high concentrations (e.g. 0.05 seconds at 0.1mM) is actually quite long at low concentrations (e.g. 14 hours at 0.1 nM). At the subnanomolar

concentrations superoxide can inactivate the citric acid cycle enzyme aconitase, can poison energy metabolism and releases potentially toxic iron. Aconitase is one of several iron-sulfur containing dehydratases in metabolic pathways shown to be inactivated by superoxide (Gardner et al., 1995). Therefore SOD is required to actively remove superoxide quickly at all concentrations. To this end SOD has one of the largest reaction rate with its substrate of any known enzyme, this reaction being only limited by the frequency of collision between itself and superoxide. Hence the SOD catalysed dismutation is favoured when concentrations of superoxide radicals are low, which occurs under physiological conditions, (Figure 1.3) and even more favoured as concentrations of superoxide become higher.

There are currently three identified members of the enzyme family, namely cytosolic Cu/Zn SOD (SOD1), mitochondrial Mn-SOD (SOD2) and extracellular SOD or ec-SOD (SOD3) (Faraci and Didion, 2004). The isoforms differ in their location, Cu/Zn SOD is found in the cytosol, Mn-SOD is localised to the mitochondria and ecSOD is found in the extracellular space. Cu/Zn SOD is the most widely distributed of these enzymes and comprises approximately 90% of the total SOD pool. The physiological importance of SODs is illustrated by the severe pathologies evident in mice genetically engineered to lack these enzymes. Mice lacking SOD2 die several days after birth, amidst massive oxidative stress (Li et al., 1995). Mice lacking SOD1 develop a wide range of pathologies, including hepatocellular carcinoma (Elchuri et al., 2005), an acceleration of age-related muscle mass loss (Muller et al., 2006), an earlier incidence of cataracts and a reduced lifespan. Mice lacking SOD3 do not show

any obvious defects and exhibit a normal lifespan, though they are more sensitive to hyperoxic injury (Sentman et al., 2006).

1.6.2 CATALASE

Catalase is an intracellular enzyme located mostly in peroxisomes but also in the cytosol. It catalyses the conversion of hydrogen peroxide to water and molecular oxygen. Thus it removes superoxide indirectly by converting the hydrogen peroxide produced by SOD. (Figure 1.3) Catalase has one of the highest turnover numbers of all enzymes. One molecule of catalase can convert millions of molecules of hydrogen peroxide to water and oxygen per second.

The true biological significance of catalase is not always straightforward to assess. Mice genetically engineered to lack catalase are phenotypically normal, indicating that this enzyme is dispensable in animals under some conditions (Ho et al., 2004). However catalase deficiency may increase the likelihood of developing Type II diabetes (Goth et al., 2001; Goth, 2008). Some human beings have very low levels of catalase (acatalasia), yet show few ill effects. As such it is likely that the predominant scavengers of H_2O_2 in normal mammalian cells are peroxiredoxins rather than catalase.

1.6.3 PEROXIREDOXINS

Peroxiredoxins are a ubiquitous family of antioxidant enzymes. They are divided into three classes namely typical 2-Cys, atypical 2-Cys and 1-Cys. These enzymes share the same basic catalytic mechanism, in which a redox-active cysteine (the peroxidatic cysteine) in the active site is oxidized to a sulfenic acid

by the peroxide substrate (Claiborne et al., 1999). The recycling of the sulfenic acid is what distinguishes the three enzyme classes. 2-Cys peroxiredoxins are reduced by thiols such as glutathione, while the 1-Cys enzymes may be reduced by ascorbic acid or glutathione (Monteiro et al., 2007).

1.6.3.1 Glutathione

Glutathione (GSH) and thioredoxin (TRX) comprise the main intracellular redox buffering capacity of which glutathione is the more abundant. Glutathione is oxidised to glutathione disulphide (GSSG) and the 2GSH/GSSG couple is a representative indicator of the redox environment of the cell (Droge, 2002; Schafer and Buettner, 2001). The high ratios of reduced to oxidised GSH and TRX are maintained by the activity of GSH reductase and TRX reductase, respectively.

Glutathione has a number of mechanisms of protecting against oxidative stress. Glutathione is a cofactor of several detoxifying enzymes against oxidative stress such as glutathione peroxidase. (Figure 1.3) Thus GSH can detoxify hydrogen peroxide and lipid peroxides by the catalytic action of glutathione peroxidase. GSH can also scavenge hydroxyl radicals and singlet oxygen directly and is also able to regenerate important antioxidants such as vitamin C and E back to their active forms (Masella et al., 2005).

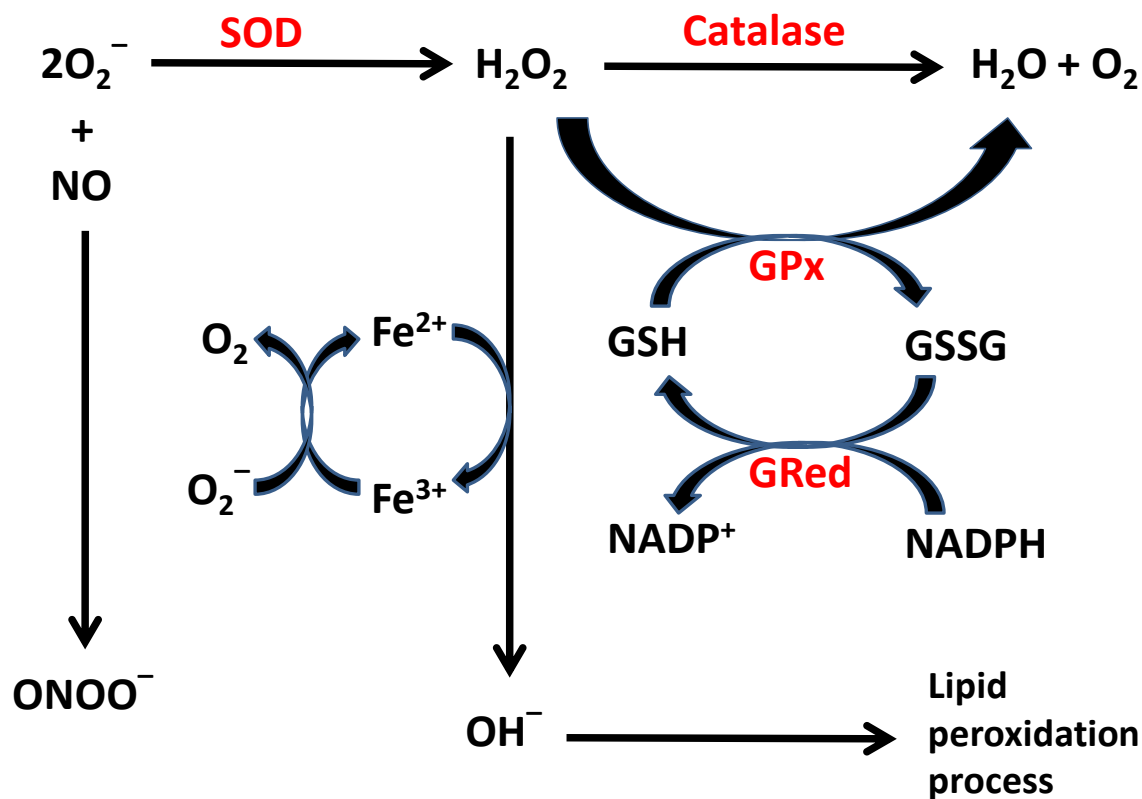


Figure 1-3 Antioxidant system

Superoxide radical is dismutated by the superoxide dismutase (SOD) to hydrogen peroxide. Hydrogen peroxide is most efficiently scavenged by the enzyme glutathione peroxidase (GPx) which requires GSH as an electron donor. The oxidised glutathione (GSSG) is reduced back to GSH by the enzyme glutathione reductase (GRed) which uses NADPH as electron donor. Some transition metals such as Fe^{2+} and Cu^+ can break hydrogen peroxide to the reactive hydroxyl radical. The hydroxyl radical are removed by their interaction in the lipid peroxidation process. Excess superoxide can react with NO to form $ONOO^-$.

1.7 NADPH OXIDASE

1.7.1 HISTORICAL OVERVIEW

NADPH oxidase is now thought to be the predominant source of ROS in vascular tissue and cardiac cells. NADPH oxidase derived ROS appears to play a physiological role in redox signaling and is the only one that appears to be specifically designed for this purpose (Cave et al., 2005; Cave et al., 2006; Geiszt, 2006; Griendling et al., 2000; Griendling, 2004; Griendling, 2006; Lambeth, 2004; Lassegue et al., 2012).

Historically, NADPH oxidase was identified in phagocytes and proposed as being responsible for the superoxide generation in the respiratory burst (ROSSI and ZATTI, 1964). A second line of evidence came from clinical findings of patients suffering from recurrent pyogenic infections. These patients had a genetic defect in their NADPH oxidase gene and no respiratory burst in their phagocytes (BERENDES et al., 1957). The genetic disorder is now referred to as chronic granulomatous disease (CGD).

The development of the cell free system allowed activation of the phagocyte NADPH oxidase to be examined using purified cytosol and membrane fractions (Bromberg and Pick, 1985; Heyneman and Vercauteren, 1984). This system provided the tool for the discovery of the cytosolic subunits p47^{phox}, p40^{phox}, p67^{phox} and the small GTP-binding proteins Rac1 and Rac2 (Abo et al., 1991; Knaus et al., 1991; Nunoi et al., 1988; Volpp et al., 1988; Wientjes et al., 1993). Progress towards understanding the phagocyte NADPH oxidase led to the observation that a similar enzyme was present in a variety of other cells

including fibroblasts, tumor cells and vascular smooth muscle cells to name a few (Griendling et al., 1994; Meier et al., 1991; Szatrowski and Nathan, 1991b). The availability of the human genome sequence resulted in the identification of the first homolog of the phagocyte NADPH oxidase termed as Nox1. In this novel Nox terminology for the enzyme the original phagocyte NADPH oxidase was termed Nox2. The identification of Nox1 was quickly followed by the cloning of Nox3, Nox4 and Nox5 and two large members of the Nox family namely DUOX1 and DUOX2 (Banfi et al., 2001; Cheng et al., 2001; De, X et al., 2000; Dupuy et al., 1999; Geiszt et al., 2000; Kikuchi et al., 2000; Shiose et al., 2001).

1.7.2 CELLULAR AND HISTOLOGICAL LOCALISATION OF NADPH OXIDASE

It is now appreciated that the NADPH oxidase isoforms have a varied tissue distribution across the body. Nox1 is highly expressed in the colon (Suh et al., 1999), Nox3 is found predominantly in the inner ear (Banfi et al., 2004a), whilst Nox4 was originally found in the kidney (Geiszt et al., 2000) but also has high expression in the vasculature and many other tissues. Nox5 is found in the lymphoid tissue and testis (Cheng et al., 2001), whereas the Duoxs have high expression levels in the thyroid gland (De, X et al., 2000). In cardiovascular tissue, expression of more than one isoform of Nox is seen in several cell types (see Table 1.1). It is increasingly thought that this implies specific roles for the different isoforms.

Table 1-1 Expression of Nox isoform mRNA in cardiovascular cells

	Cardiomyocytes	Endothelial cells (EC)	Fibroblasts	Vascular smooth muscle cells (VSMC)
Nox 1	-	Human coronary artery EC (Sorescu et al., 2002) Rat aortic EC (Ago et al., 2004) Rat basilar artery EC (Ago et al., 2005)	Human cardiac fibroblasts (Sorescu et al., 2002)	Human coronary artery SMC (Sorescu et al., 2002) Rat aortic SMC (Lassegue et al., 2001) Mouse aortic SMC (Grote et al., 2003) Rabbit pulmonary artery SMC (Weissmann et al., 2000) Rat mesenteric artery SMC (Touyz et al., 2002)
Nox 2	Mouse cardiomyocytes (Bendall et al., 2002a; Peng et al., 2005) Rat cardiomyocytes (Xiao et al., 2002)	Human coronary artery EC (Sorescu et al., 2002) Rat aortic EC (Ago et al., 2004) Rat basilar artery EC (Ago et al., 2005)	Human cardiac fibroblasts (Sorescu et al., 2002) Adventitia of human coronary artery (Sorescu et al., 2002) Adventitia of mouse aorta (Wang et al., 2001)	Human coronary artery SMC (Sorescu et al., 2002) Rat aortic SMC (Lassegue et al., 2001)
Nox 4	Mouse cardiomyocytes (Peng et al., 2005)	Human coronary artery EC (Sorescu et al., 2002) Rat aortic EC (Ago et al., 2004) Rat basilar artery EC (Ago et al., 2005)	Human cardiac fibroblasts (Cucoranu et al., 2005; Sorescu et al., 2002) Adventitia of human coronary artery (Sorescu et al., 2002) Rat cardiac fibroblasts (Colston et al., 2005)	Human coronary artery SMC (Sorescu et al., 2002) Rat aortic SMC (Lassegue et al., 2001) Rat mesenteric artery SMC (Touyz et al., 2002)
Nox 5	-	Human EC ((Belaiba et al., 2007))	Human cardiac fibroblasts (Cucoranu et al., 2005)	Human aortic SMC (Pedruzzi et al., 2004) SMC within human aortic atherosclerotic lesions (Kalinina et al., 2002)

1.7.3 NADPH OXIDASE STRUCTURE

NADPH oxidases are transmembrane proteins that transport electrons across biological membranes to reduce oxygen to superoxide. Nox2 is the prototype NADPH oxidase and has been the most extensively studied.

The prototypical NADPH oxidase complex consists of a membrane-bound flavocytochrome b_{558} , which is a heterodimer consisting of a catalytic Nox subunit and $p22^{phox}$ (Babior, 2004). The membrane bound Nox subunit transfers electrons from NADPH, in the phagocyte cytoplasm, across two heme units, to molecular oxygen, thereby producing superoxide (Cross et al., 1995). Although the Nox subunit is the catalytic component of the flavocytochrome b_{558} , its partner $p22^{phox}$ subunit is also essential. The expression of $p22^{phox}$ subunit and the formation of the heterodimer is a prerequisite for its egress from the endoplasmic reticulum and proper localisation in the plasma membrane. Consistent with the requirement of heterodimer formation for stability is the observation that patients with genetic defects in either Nox or $p22^{phox}$ subunits lack both proteins (Heyworth et al., 2003). Subunit $p22^{phox}$ is required by Noxes 1, 2, 3 and 4 for protein stabilisation and for binding of regulatory cytosolic subunits.

There are several cytosolic subunits namely $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and the small GTPase Rac1 or Rac2 are known to be involved in the activation of Nox2. (Figure 1.4) Biochemical studies using broken cell system have demonstrated that $p47^{phox}$ serves as an organising adaptor protein lacking intrinsic catalytic activity. However $p67^{phox}$ represents an essential activating cofactor which possesses the domain that regulates the reduction of FAD by

NADPH (Nisimoto et al., 1999). Similarly to Nox2, other Nox isoforms have associated factors. Homologues of p47^{phox} and p67^{phox} named NOX organising (NOXO1) and activating (NOXA1) proteins have been identified and each implicated in the activity of NOX1 and 3 (Bedard and Krause, 2007). In contrast to Nox2, Nox4 does not require the cytosolic subunits. Therefore p22^{phox} is the only subunit essential for Nox4 activation. This may explain why Nox4 appears to be constitutively active (Ambasta et al., 2004) with its activity suggested to be transcriptionally regulated (Serrander et al., 2007). This would therefore negate a requirement for control by regulatory cytosolic subunit proteins. For Nox5 and Duox proteins calcium serves as a regulator (Banfi et al., 2004b; Banfi et al., 2001; Lambeth et al., 2007; Nisimoto et al., 1999).

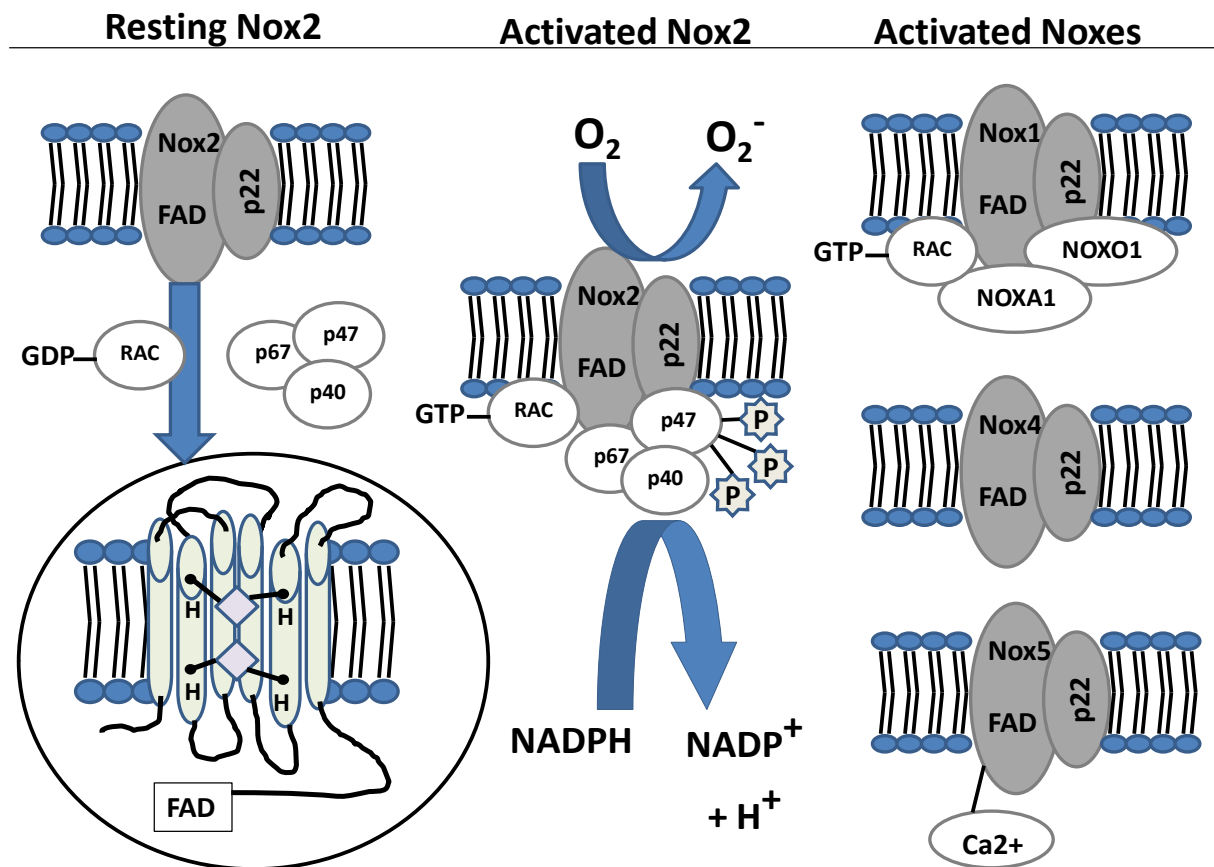


Figure 1-4 Structure and activation of NADPH oxidase

In resting cells, heterodimeric Nox and p22^{phox} resides in the membrane, whereas the complex of p47^{phox}, p67^{phox} and p40^{phox} is cytosolic. The Nox enzyme is shown in more detail in the circled inset. The Nox enzyme has six transmembrane domains. Two of these contain two histidines (H), coordinating two hemes (purple squares). The cytoplasmic COOH terminus contains FAD and NADPH binding domains. The Nox family of enzymes differ in their mechanism of activation. For Nox2, agonists trigger the phosphorylation of the autoinhibitory domain of p47^{phox}, releasing a conformational restriction allowing for its association at the membrane. Nox1 and 3 require NOXO1 and NOXA1 whereas Nox4 is constitutively active without the requirement for other subunits. Nox5 is activated by calcium and does not appear to require subunits.

1.7.4 ACTIVATION AND FUNCTION OF NADPH OXIDASES

The activation of Nox2 happens through a complex series of protein-protein interaction. Nox2 constitutively associates with p22^{phox} and its activation requires the translocation of cytosolic factors to the Nox2/p22^{phox} complex. The phosphorylation of the autoinhibitory region in p47^{phox} leads to a conformational change in the p47^{phox} allowing for it to interact with p22^{phox} (Groemping et al., 2003; Sumimoto et al., 1996). The region of p22^{phox} responsible for this interaction is the COOH terminus (Leto et al., 1994; Sumimoto et al., 1996). Truncation of p22^{phox} or mutations within the COOH terminus domain leads to a loss of activation of Nox1, Nox2 and Nox3 (Kawahara et al., 2005; Leusen et al., 1994). In agreement with the concept that Nox4 activation does not involve cytosolic subunits, these truncations or mutations of p22^{phox} COOH terminus does not decrease Nox4 activity.

Interestingly AA has been reported to activate NADPH oxidase directly by stimulating p22^{phox}, thereby inducing ROS generation (Kim and Dinanuer, 2006; Luchtefeld et al., 2003; Shiose and Sumimoto, 2000). LOX-and COX-generated AA metabolites have also been demonstrated to stimulate the production of ROS by NOX thus revealing the existence of an inter-connected signalling system between eicosanoids and NOXs (de Carvalho et al., 2008; Luchtefeld et al., 2003). For example, a 5-LOX metabolite, leukotriene B₄ (LTB₄) and COX metabolite, PGF₂α, are capable of stimulating NADPH oxidase. This LTB₄ induced ROS generation occurring via NOX has been seen to be crucial in cell chemotaxis (Woo et al., 2002; Woo et al., 2003).

The localisation of p47^{phox} to the membrane brings p67^{phox} and p40^{phox} into contact with Nox2. The subunits p67^{phox}, p40^{phox} and Rac fail to translocate to the membrane in neutrophils from patients lacking p47^{phox} (Dusi et al., 1996; Heyworth et al., 1991; Kawahara et al., 2005).

The protein kinase C (PKC) isoforms β , δ and ϵ are suggested to be the major kinases responsible for p47^{phox} phosphorylation but recent studies suggest that other kinases such as Akt, , p38MAPK, and p21 activated kinase (PAK) can also be involved. The precise role of p40^{phox}, which has significant homology to p47^{phox}, in oxidase activation is poorly understood.

Interestingly, recent data indicate that p47^{phox} may have additional roles in nonphagocytic cells. It has been suggested that protein–protein interactions involving p47^{phox} and other nonoxidase factors may play an important role in the spatial confinement of NADPH oxidase. Thus this allows for locally derived ROS to modulate signals and thereby enabling local redox signaling. Several different proteins have been found including the TNF receptor-associated factor 4 (TRAF4) and WAVE1, an important regulator of the cytoskeleton, which may act as a scaffold to enable the recruitment of the NADPH oxidase. Such local redox modulation by NADPH derived ROS has been seen to be involved with cytoskeletal regulation (Li et al., 2005; Xu et al., 2002) and therefore in cellular migration.

Rac also becomes activated from GDP to GTP, catalysed by guanine nucleotide exchange factors (GEFs) such as Tiam-1, Trio, and Vav-1 and P-Rex1, which appears to be of particular importance (Mizrahi et al., 2005; Price et al., 2002; Welch et al., 2002). The GTPase Rac interacts directly with Nox2 followed

by a subsequent interaction with p67^{phox} thus enabling optimal positioning of Rac and completing the assembly. Once assembled the complex is active and generates superoxide by transferring an electron from NADPH in the cytosol to oxygen on the luminal or extracellular space.

The electrons are transferred in a series of steps. The electrons are transferred from the NADPH to FAD, a process that is regulated by p67^{phox} (Nisimoto et al., 1999). A single electron is then transferred from the reduced flavin FADH₂ to the iron centre of the inner heme. Since the iron of the heme can only accept one electron, the inner heme must donate its electron to the outer heme before the second electron can be accepted from the now partially reduced flavin FADH. Oxygen is bound to the outer heme as a final step to accept the electron (Cross and Segal, 2004; Doussiere et al., 1996; Vignais, 2002). The process is electrically balanced by proton conduction in the same direction (Lassegue and Clempus, 2003).

1.8 INVOLVEMENT OF NOX2 IN CARDIAC HYPERTROPHY

Studies of hypertrophy in isolated cultured cardiomyocytes have identified an important role for intracellular ROS generation. As such Hirotani et al (Hirotani et al., 2002) showed that the GPCR agonists (AngII, endothelin-1, phenylephrine) induced cardiac hypertrophy was mediated through NF-κB activation via the generation of ROS. Nakamura et al (Nakamura et al., 1998) also showed in cardiac myocytes that TNF-α and AngII caused hypertrophy via the generation of ROS. Similarly Xiao et al (Xiao et al., 2002) provided the

evidence that ROS from NADPH oxidase appeared to be involved in α_1 -adrenoceptor stimulated cardiomyocyte hypertrophy.

Definitive evidence for the involvement of NADPH oxidase in pathological hypertrophy came from experiments involving genetically modified mice. Global Nox2^{-/-} mice subjected to subpressor dose of AngII for 2 weeks showed significantly reduced cardiac hypertrophy compared to wild type (Bendall et al., 2002a). This result suggested that AngII induced cardiac hypertrophy, in the absence of a pressor response, requires Nox2 oxidase activity. In line with this finding, AngII induced hypertrophy of isolated cardiomyocytes were shown to involve the activation of Nox2 oxidase (Hingtgen et al., 2006a). As Nox2^{-/-} mice have a lower baseline blood pressure than wild type, it is possible that this may have contributed to the difference seen by Bendal et al (Bendall et al., 2002a). Hence Byrne et al (Byrne et al., 2003) investigated the effect of a pressor dose of AngII in inducing cardiac hypertrophy. These experiments again showed a reduced level of cardiac hypertrophy in the Nox2^{-/-} mice again suggesting that independent of the mechanical effect of blood pressure, chemical stimulation by angiotensin II induces cardiac hypertrophy via Nox2 oxidase.

In contrast to AngII induced hypertrophy, the hypertrophic response to aortic banding was found to be similar between Nox2^{-/-} and wild type mice by Byrne et al (Byrne et al., 2003). They also found an increased level of NADPH oxidase activity which they ascribed to the increased levels of Nox4 seen in the Nox2^{-/-} mice. Maytin et al have also shown that pressor effects from aortic banding induced similar levels of cardiac hypertrophy in both wild type and

Nox2^{-/-} mice (Maytin et al., 2004). These authors also found increased levels of p22^{phox} and p47^{phox} mRNA. Taken together these data support the fact Nox2 does not play an essential role in cardiac hypertrophy as induced by the mechanical effect of aortic banding. The data also suggests that Nox4, as the source of ROS, could be important in the development of observed cardiac hypertrophy.

1.9 NADPH OXIDASE DERIVED ROS AND CARDIAC FIBROSIS

A significant body of data implicates Nox2 in the development of cardiac fibrosis. As such, our laboratory has shown that Nox2^{-/-} mice had a markedly reduced fibrosis compared to wild type animals in response to either a subpressor or pressor infusion of AngII (Bendall et al., 2002a; Byrne et al., 2003; Johar et al., 2006). Furthermore the profibrotic effects of AngII have been shown to be inhibited by the antioxidant N-acetyl-cysteine, consistent with the role of ROS in this process (Johar et al., 2006).

Nox2 has also been implicated in aldosterone induced myocardial fibrosis (Johar et al., 2006). Aldosterone infusion resulted in NADPH oxidase activation and increased cardiac fibrosis, with these effects being inhibited in Nox2^{-/-} mice. The authors also provided evidence that mineralocorticoid receptor (MR) activation is involved in AngII induced myocardial NADPH oxidase activation and subsequent interstitial cardiac fibrosis. In line with this, spironolactone inhibited both the increase in NADPH oxidase activity.. This finding suggested that MR receptor activation was important in the AngII stimulation. In a more recent study, apoptosis signal-regulating kinase 1 (ASK1) deficient mice

subjected to aldosterone/salt treatment showed a marked reduction in Nox2 associated ROS production with a significant reduction in interstitial fibrosis and perivascular fibrosis (Johar et al., 2006; Nakamura et al., 2009).

Johar et al found that interstitial cardiac fibrosis induced by AngII and aldosterone involved the Nox2 dependent upregulation of profibrotic cytokines such as CTGF and profibrotic genes such as pro-collagen-1, pro-collagen-3 and fibronectin. They also found that there was increased Nox2 dependent activation of NF- κ B and MMP2 (Johar et al., 2006; Nakamura et al., 2009).

In contrast to the above mentioned role of Nox2, some recent studies suggest an important role of Nox4 in the development of cardiac fibrosis. Cucoranu et al in a study involving cultured human cardiac fibroblasts found that Nox4 and 5 mRNAs were abundantly expressed (Cucoranu et al., 2005). These authors reported that TGF- β_1 upregulated Nox4 expression and promoted the conversion of fibroblasts to myofibroblasts which are the main producers of collagen in the extracellular matrix. In line with the role for Nox4 in fibrosis Byrne et al (Byrne et al., 2003) also demonstrated an increase in Nox4 protein expression following AngII infusion.

The evidence for cardiac fibrosis secondary to the pressor effect of aortic banding is conflicting. Matyn et al (Maytin et al., 2004) published evidence that Nox2 oxidase did not play an important role in cardiac fibrosis in this model as myocardial fibrosis was increased to a similar level in both wild type and Nox2^{-/-} mice. In contrast Greive et al (Grieve et al., 2006) demonstrated a reduced level of fibrosis in the Nox2^{-/-} mice implicating an important role for the Nox oxidase in myocardial fibrosis following aortic banding.

In summary the data would suggest an important role of Nox2 in AngII induced cardiac fibrosis independent of any pressor effect. The activation of the MR is also important in this AngII induced cardiac fibrosis. More recently Nox4 has been found to be important in the development of cardiac fibrosis, the expression of the oxidase being upregulated by both TGF- β_1 and AngII. The role of Nox2 is conflicting in a mechanical pressure overload model as seen after aortic banding.

1.10 CELLULAR SOURCE OF NOX2

The development of cardiac fibrosis is a complex process involving the interactions of multiple cells. The evidence in support for the common dependence on Nox2 for the development of cardiac fibrosis in several cardiac pathologies has been suggested (Grieve et al., 2006; Johar et al., 2006; Looi et al., 2008). A key question is how Nox2 drives this cardiac fibrosis. Nox2 is expressed in cardiac fibroblasts, endothelial cells and in monocytes/macrophages, and an obvious possibility is that the cellular expression of Nox2 in any of these cell types may be an important factor.

For example the Nox2 in circulating monocytes could be important in driving the conversion of the monocyte phenotype to a pro-inflammatory one which encourages cardiac infiltration. Conversely endothelial production of ROS may also be important with respect to endothelial activation and the attraction of pro-fibrotic inflammatory cells (Grafe et al., 1997; Li and Shah, 2003; Li and Shah, 2004; Pueyo et al., 2000). As such it is known that the adhesion of circulating monocytes to the endothelium of the vessel wall and the subsequent

migration of the cell into the heart is an essential step in the development of cardiac fibrosis (Kuwahara et al., 2003). Once in the heart, macrophages can release cytokines to stimulate the transformation of fibroblasts to myofibroblasts and thus increased production of collagen, a pivotal step in the development of cardiac fibrosis (Weber et al., 1993). Nox2 in the fibroblast could be critical for the transformation of the fibroblast to an activated phenotype. The cell-specific contribution of Nox2 remains to be established, in which the activation of Nox2 is critical for the development of the fibrotic process.

1.11 Aim

The aims of this thesis were:

- To investigate the role of Nox2 in the redox response of cardiac fibroblasts.
- To investigate the role of Nox2 in macrophage migration and their circulating inflammatory profile.
- To investigate the contribution of Nox2 in circulating inflammatory cells versus resident cardiac cells towards Ang II induced cardiac hypertrophy and fibrosis.

2 ROLE OF NOX2 IN ANGIOTENSIN II STIMULATED ROS RESPONSE IN CARDIAC FIBROBLASTS

2.1 INTRODUCTION

During normal cardiac function cellular components of the heart interact in a dynamic fashion to respond to changes in development, homeostatic and pathological stimuli. The main cellular constituents of the heart include cardiac fibroblasts, myocytes, endothelial cells and vascular smooth muscle cells with the majority of the cells consisting of fibroblasts and cardiac myocytes (Camelliti et al., 2005). Cardiac myocytes occupy approximately 75% of normal myocardial tissue volume, but they account for only 30–40% of cell numbers (Baudino et al., 2006; Vliegen et al., 1991). The majority of the remaining non-myocyte cells are fibroblasts. Fibroblasts are found throughout the cardiac tissue, surrounding myocytes and contribute to the structural, biochemical, mechanical and electrical properties of the myocardium (Kohl and Noble, 1996; MacKenna et al., 2000; Sun et al., 2002a). Moreover these cells help maintain the ECM homeostasis of the heart via the autocrine and paracrine action of secreted factors as well as via direct cell-cell interaction (Baudino et al., 2006; Camelliti et al., 2004; Camelliti et al., 2005; Gaudesius et al., 2003; Manabe et al., 2002; Sussman et al., 2002). Alterations in these signals or biochemical input can cause deleterious, adaptive and/or compensatory changes in the heart. Cardiac fibrosis is frequently associated with myocardial remodelling as in ischemic and rheumatic heart disease, inflammation, hypertrophy, and infarction. The increase in fibrous tissue content and the synthesis of extra-cellular matrix

(ECM) proteins is reliant on the proliferative and secretory potential of fibroblasts.

2.1.1 IDENTIFICATION OF FIBROBLASTS

Traditionally fibroblasts are defined as cells of mesenchymal origin that produce interstitial collagen and have classically been viewed as a uniform cell type. This view has been challenged as fibroblasts have been shown to demonstrate extensive phenotypic heterogeneity according to the different tissue and the physiological condition of the tissue from which they are harvested (Chang et al., 2002; Fries et al., 1994; Lekic et al., 1997). Morphologically cultured fibroblasts are flat spindle shaped cells with multiple processes emanating from the main cell body. They tend to display a prominent Golgi apparatus and extensive rough endoplasmic reticulum especially when active. The lack of a truly specific marker has long been a limiting factor in studying fibroblasts in vivo. The intermediate filament vimentin is abundant in cardiac fibroblasts and has been used as a marker to identify cardiac fibroblasts (Camelliti et al., 2005). However this marker is also present in other cell types, for example vascular endothelial cells. Nevertheless given the characteristic cyto-morphological differences between these cell types, vimentin has been a suitable tool for reliable identification of cardiac fibroblasts.

A more specific marker for cardiac fibroblasts is the collagen receptor Discoidin Domain Receptor 2 (DDR2) (Goldsmith et al., 2004). DDR1 and DDR2 represent a relatively novel family of collagen specific receptor tyrosine kinases involved in the conversion of extracellular stimuli into cellular

responses. These receptors mediate a variety of cell functions, including growth, migration, morphology and differentiation. The tissue distribution of DDR1 and DDR2 varies and can be mutually exclusive. DDR2 expression has been detected in both rat and mouse heart. Originally defined as a collagen receptor on mesenchymal cells, DDR2 has also been found on leukocytes, as well as in tumours, but importantly not on cardiomyocytes, cardiac endothelial or smooth muscle cells (Camelliti et al., 2005; Goldsmith et al., 2004). Therefore, in the heart, the DDR2 receptor is fibroblast specific. Another marker that has been proposed to be fibroblast specific is fibroblast-specific-protein-1. However studies in the literature have shown FSP-1 to be expressed in other cells including leukocytes (Mazzucchelli, 2002).

2.1.2 MYOFIBROBLASTS AS NOX2 EXPRESSING CELLS

Fibroblasts are phenotypically heterogeneous and under appropriate conditions, resting or quiescent fibroblasts can acquire an active, synthetic, contractile phenotype termed myofibroblast. The myofibroblasts express several smooth muscle cell markers that are not typically expressed in fibroblasts (Tomasek et al., 2002). These myofibroblasts are more mobile than fibroblasts and can contract collagen gels and are therefore thought to be important in wound closure of healing scars (Sun and Weber, 2003). Indeed research has shown that myofibroblasts play a key role in the reparative fibrosis following myocardial infarction (Calderone et al., 2006).

Fibroblasts can respond to a wide range of different stimuli including hypoxia, hormonal (e.g AngII) and mechanical (e.g. stretch). TGF β ₁, cytokines

and other growth factors (Kaden et al., 2005; Serini et al., 1998; Tamaoki et al., 2005; Walker et al., 2004) have been shown to transform fibroblasts to myofibroblasts. In keeping with this, stimulation of cardiac fibroblasts by TGF β_1 has also been shown to increase the synthesis of collagen, fibronectin, and proteoglycans (Dobaczewski et al., 2010; Eghbali et al., 1991; Heimer et al., 1995; Leask, 2007; Villarreal et al., 1996).

In global knockout experiments, ROS and more specifically Nox2 was shown to be important in the process of aldosterone or AngII induced cardiac fibrosis or pressor induced hypertrophy (Bendall et al., 2002a; Byrne et al., 2003; Johar et al., 2006). However the exact cell source of the Nox2 critical in this process was not identified. Loss of Nox2 in a range of different cell types could be important including cardiac myocytes, endothelial cells, fibroblasts or macrophages. In particular, Nox2 in cardiac fibroblasts could be an important cell source. AngII is the effector molecule of the RAS, and cardiac fibroblasts can express angiotensinogen, renin and ACE, allowing them to produce AngII locally in the heart (Sanghi et al., 2005). Also, AngII stimulation has been shown to increase collagen production and secretion from cardiac fibroblasts (Autelitano et al., 2003; Lijnen et al., 2001). As stimulation by AngII is known to be mediated by Nox2, cardiac fibroblasts could be a possible cell source of the Nox2, the loss of which in AngII stimulated mice resulted in a loss of fibrosis and hypertrophy. In this chapter, the ROS response mediated by Nox2 in cardiac fibroblasts following AngII stimulation is investigated.

2.1.3 *AIM*

The aims of this chapter were to develop an *in vitro* model using murine primary cardiac fibroblasts to investigate the role of the Nox2 isoform of NADPH oxidase in AngII induced ROS production.

2.2 METHODS

2.2.1 CREATION OF *NOX2*^{-/-} MICE

Experiments were conducted in WT and *Nox2*^{-/-} male littermate controls on a C57BL/6J background. *Nox2*^{-/-} mice have previously been reported as a model of chronic granulomatous disease by homologous recombination and have a neomycin resistance cassette inserted in exon 3 of the *Nox3* subunit on the X chromosome. Phagocytic cells from *Nox2*^{-/-} mice do not exhibit an oxidative burst after stimulation with phorbol myristate as compared with WT mice. However there is no obvious phenotype apart from an increased susceptibility to certain infection (Pollock et al., 1995).

These mice were originally obtained from the Jackson Laboratory and a colony bred at the Comparative Biology Unit at Kings College London. Subsequently, the animals were backcrossed with the C57BL/6J strain established in the unit to minimize possible general variability. Determining the phenotype to confirm the presence or absence of the *Nox2* gene, in isolated genomic DNA from each individual mice, genotyping was performed. Ear punches harvested under anesthesia (2.5% isoflurane/97.5% Oxygen (2L/min)) were digested in a solution of Proteinase K (10mg/ml) Roche, UK) in an alkaline buffer (0.1M Tris EDTA, 0.1M NaEDTA, 1%SDS and 0.15M NaCl, pH 8.0) for 2-4 hours at 55°C cDNA was precipitated in isopropanol, washed with 80% ethanol and resuspended in TE buffer (imMEDTA, 10mM Tris pH 8.0). Amplification of resultant cDNA was conducted in a thermal cycler (AB Applied Biosystems, (UK) involving 35 cycles of denaturing (30sec at 94°C), annealing

(30sec at 56°C) and synthesis (30sec at 72°C). A duplex reaction reaction on 1µL of DNA was achieved using primers (10µM) for Nox2 in a 25µL total reaction volume containing 12.5µL Red Taq Ready Mix, 0.5µL forward primer, 0.5µL reverse primer1, 1µL of reverse primer2 (Table) and 9.5µL nuclease free water. Subsequent polymerase chain reaction (PCR) products were separated by electrophoresis on a 1.5% agarose gel containing Ethidium Bromide (400ng/mL) in 40 mM Tris-acetate, 1 mM EDTA and visualised under ultraviolet light. A single band at ~240basepairs (bp) confirmed WT status, a single band at ~195bp confirmed a Nox2^{-/-}, whereas a double band at ~240bp and 195bp respectively confirmed heterozygote.

2-1 Primer used in genotyping

Primer sequence	
Forward	AAG AGA AAC TCC TCT GCT GTG AA
Reverse1	CGC ACT GGA ACC CCT AGA AAA GG
Reverse2	GTT CTA ATT CCA TCA GAA GCT TAT CG

2.2.2 HARVESTING PRIMARY CARDIAC FIBROBLAST BY COLLAGENASE DIGESTION

Mice were heparinised and terminally anaesthetised with pentobarbitone sodium by intraperitoneal injection. Following a midline incision of the thorax, hearts were excised and transferred immediately into cold, sterile-filtered Krebs Henseleit buffer (KHB, containing NaCl 120mM, KCl 5.7mM, NaH₂PO₄ 1.38mM, MgSO₄ 0.66mM, Glucose 10mM, HEPES 20mM, pH 7.2 – 7.4). Hearts were then rinsed twice in KHB and transferred to a dry petri dish. 5 drops of Liberase enzyme (1mL Liberase Blendzyme IV, 200µl 2.5% Trypsin, 360µl

HEPES 1M, 35 μ l Hank's Balanced Salt Solution) was added and heart tissue was finely minced before it was transferred into a 20ml glass bottle. 4-5ml of Liberase was added to the chopped heart tissue in the bottle and placed in a warm (37°C) shaking water bath for 10 minutes. The bottle was then removed and allowed to stand. Once the undigested tissue had settled, the supernatant was aspirated, added to 10mls of cold, sterile KHB and placed on ice. 2-3mls of fresh Liberase was added to the remaining tissue and replaced in the shaking water bath for a further 5 minutes of digestion. The last step was repeated until all the heart tissue had been digested typically taking 5-6 cycles. The tubes containing the tissue digest and KHB were centrifuged at 1200rpm, at 40C for 5 minutes. The pellet was then resuspended in 10mLs of KHB and spun down again. The supernatant was again removed and the primary cardiac fibroblast (PCF) cells resuspended in 5mL of Dulbecco's modified Eagle's medium, (DMEM, with 10% foetal calf serum FCS, 5% Penicillin/Streptomycin, 5% L-Glutamine). The PCFs were then placed into T75 tissue culture flasks that had been pretreated with 1% gelatin for 1 hour, and incubated in a CO₂ incubator for the cells to attach and grow for 12 hours. The PCFs were passaged in a 1:2 ratio and used at passage 2. The cells were passaged by washing the flasks with PBS and detaching the cells with 0.25% trypsin (with EDTA). The trypsin was neutralised with FCS and the cells pelleted by centrifuging at 1200rpm for 5 minutes. The pelleted cells were resuspended in fresh DMEM media and placed into a newly prepared T75 flask.

2.2.3 IMMUNOCYTOCHEMISTRY OF PRIMARY CARDIAC FIBROBLASTS

Five thousand passage 2 WT PCFs were plated onto glass bottomed LabTek chamber slides (precoated with 1% gelatin), fixed with 10% methanol, and blocked with 5% goat serum and 3% BSA. Cells were incubated with primary antibody for 90mins at room temperature. (Table 2.1) A anti-DDR2 primary antibody (Santa Cruz, USA) for tyrosine kinase receptor for collagen was used to positively identify fibroblasts. The secondary antibody was incubated for 30mins and the slides viewed under a Zeiss Axioskop 2 microscope and images taken with appropriate filters. The endothelial cell specific marker von Willebrand factor (vWF) was used to identify endothelial contamination.

Table 2-2 Primary and secondary antibodies for immunocytochemistry

	Primary	Secondary
DDR2	Rabbit anti-DDR2 (1:10)	Alexa 488 conjugated goat anti-rabbit IgG (1:200)
vWF	Rabbit anti-vWF (1:200)	Alexa 488 conjugated goat anti-rabbit IgG (1:200)

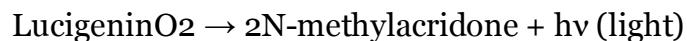
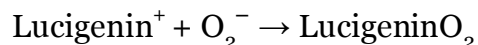
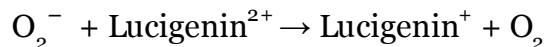
2.2.4 FLOW CYTOMETRY OF PRIMARY CARDIAC FIBROBLASTS

WT PCF from passage 0 and passage 1 were used to assess the purity of the cell isolation with FACS. Samples of 100,000 cells were suspended in cold PBS and kept on ice. Cells were positively identified by incubation with an

antibody against the fibroblast surface antigen CD90, conjugated with fluorescein-isothiocyanate (FITC) (1:10 concentration, 30mins incubation). To identify if there were any endothelial cells present in the isolation, the cells were incubated with an antibody against endothelial cell-specific vWF (1:200, 30mins). Samples were assayed in the FACSVantage machine (Becton Dickinson) using an excitation wavelength of 488nm and emission wavelength of 510nm along with the appropriate non-specific IgG controls. 10,000 events per sample were recorded. The counts (cell number) were plotted against fluorescence (Long scale) using CellQuest software.

2.2.5 CHEMILUMINESCENCE MEASUREMENT OF ROS PRODUCTION

Assessment of changes in superoxide production in PCF from aldosterone or angiotensin stimulation were undertaken using lucigenin enhanced chemiluminescence (LEC). Luminometric assays, among other techniques, provide a sensitive and fast method to detect superoxide anion (O_2^-) (Brandes and Janiszewski, 2005). Levels of superoxide production from PCF were determined using the LEC with a Lucy 1 luminometer machine (Rosys Anthos, Austria). Lucigenin is an acridylum dinitrate compound that emits light on reduction by interaction with superoxide anion. The relevant reactions of the assay are as follows



The flux of photons (hν) emitted is measured in terms of LEC intensity and taken as representing superoxide anion levels, especially at low lucigenin concentration (5μM). High concentrations (200 μM) of lucigenin can cause a large increase in LEC by itself, partially due to redox cycling (Munzel et al., 2002; Pagano, 2001) NADPH (300μM) is added to maximise O₂⁻ production associated with the NADPH oxidase (Brandes and Janiszewski, 2005; Munzel et al., 2002).

2.2.5.1 Homogenate Method

PCFs from WT and Nox2^{-/-} mice were plated onto 10cm Petri dishes (60cm²) in DMEM (10% FCS & 25mM glucose). The plates were pre-coated in 1% gelatine/PBS and seeded at 300,000 cells/dish and allowed to settle overnight. Cells were serum starved for 24 hours with serum free DMEM (5mM glucose). After 24hrs of serum starvation the appropriate stimulation was added for a further 24hrs. The medium was removed and the plate washed with PBS and the cells dislodged by scraping in 400μl of buffer B (50mM monobasic potassium phosphate, 1mM EGTA, 150mM sucrose, pH 7.0) with 2μl of protease inhibitor cocktail. The cells were homogenised with a Polytron PT2100

homogeniser for 10 second bursts. All samples were kept on ice and their protein concentrations determined using the Bradford colorimetric technique.

Ten μg of the cell homogenate sample was loaded onto a 96 well microplate containing buffer B to make the total volume $75\mu\text{L}$. Arachadonic acid ($30\text{ }\mu\text{M}$) was added for 5 minutes as a positive control to demonstrate cellular ROS production in response to physiological stimulation. Inhibitors were also added 5 minutes prior to the addition of NADPH. ROS production was stimulated by adding NADPH ($300\mu\text{M}$) to each well followed by dark-adapted lucigenin ($5\mu\text{M}$ final conc). Readings were taken every minute for 20-30 minutes at 37°C .

Bradford assay

A standard bovine serum albumin (BSA) concentration curve was generated. $950\mu\text{l}$ of a 1 in 5 dilution of BioRad dye reagent from the BioRad assay kit (BioRad Laboratories, Germany) was added to $50\mu\text{l}$ of BSA standards ($0 - 0.5\mu\text{g}/\mu\text{l}$). Light absorbance was measured by a spectrophotometer (Ultrospec 2000, Pharmacia Biotech, UK at 595nm) and a standard curve generated.

Inhibition of NADPH

The enzymatic source of NADPH-stimulated LEC in the PCF was determined pharmacologically using the following inhibitors in Table 2.2 with 5-min preincubation.

Table 2-3 Inhibitors used to determine the source of ROS production

	INHIBITORS	SITE OF ACTION
1)	Diphenylene iodonium (DPI) (10 μ M)	Inhibits all flavoprotein including NADPH oxidase
2)	Rotenone(2 μ M)	Mitochondrial respiratory chain complex I inhibitor
3)	Nitro-L-arginine methyl ester (L-Name) (100 μ M)	Nitric oxide synthase inhibitor
4)	Thenoyltrifluoroacetone (TTFA) (10 μ M)	Mitochondrial respiratory chain complex II inhibitor
5)	Allopurinol(100 μ M)	Xanthine oxidase inhibitor
6)	Tiron(16mM)	Superoxide scavenger

2.2.5.2 Adherent whole-cell method

WT and Nox2^{-/-} PCFs from passage 1 were seeded onto gelatin-coated 96 well microplates (5000 cells/well) in DMEM with 0.5% serum and left to settle overnight. The cells were incubated with appropriate stimulation, namely AngII (10⁻⁷ M) for 24 hrs. Following stimulation the cells were washed with PBS and

75µL of intracellular (IC) solution (140mM KCL, 1 mM EGTA, 6 mM glucose, 20mM K-PIPS, 1 mM MgCl₂, pH 7) was placed into the wells. To enable NADPH and lucigenin to enter the cells, the PCFs were permeablised by adding 2µL of 0.5% Saponin to each well. AA (30 µM) was added 5 mins prior to the addition of NADPH as a positive control to show the cells were viable and able to respond. ROS production was stimulated by adding NADPH (300µM) to each well followed by dark-adapted lucigenin (5µM). Readings were taken every minute for 20-30 minutes at 37°C.

2.2.6 REAGENTS

All the reagents in this thesis were obtained from Sigma , UK unless otherwise specified.

2.2.7 STATISTICAL ANALYSIS

NADPH-stimulated LEC was measured for 20mins. LEC increased over the first 10-15 mins and then gradually plateaued. As such the area under the curve (AUC) in arbitrary units represented the cumulative LEC over 20mins. The values are presented as means +/- SEM. Statistical significance was investigated by fitting regression models to the AUC data. STATA statistical software package was used and a p-value of less than 0.05 was considered significant.

2.3 RESULTS

2.3.1 *CHARACTERISATION OF FIBROBLASTS*

2.3.1.1 **Bright field and immunocytochemistry**

The light field image (Figure 2.1) showed no cardiac myocytes as they have been destroyed in the collagenase digestion process. The majority of the cells are cardiac fibroblasts contaminated with some endothelial cells. Cardiac fibroblasts appeared spindle-like in shape and spread out. (Figure 2.1A, B & C) In comparison the endothelial cells were smaller in size and clump together to form cobblestone-like patches. (Figure 2.1C) The shapes of the WT PCFs varied, consistent with fibroblasts being phenotypically heterogenous.

PCFs were identified with DDR2 staining (Figure 2.2 A & B) and compared to that of the 3T3 fibroblast cell line (Figure 2.2 C). Both cells showed the speckled staining of DDR2. Assessment of several slides under low power showed that majority of the cells were cardiac fibroblasts. Fibroblasts stained with non-specific IgG negative control showed little background staining. A small number of contaminating endothelial cells were identified by staining for the endothelial specific cell marker vWF (Figure 2.2 D)

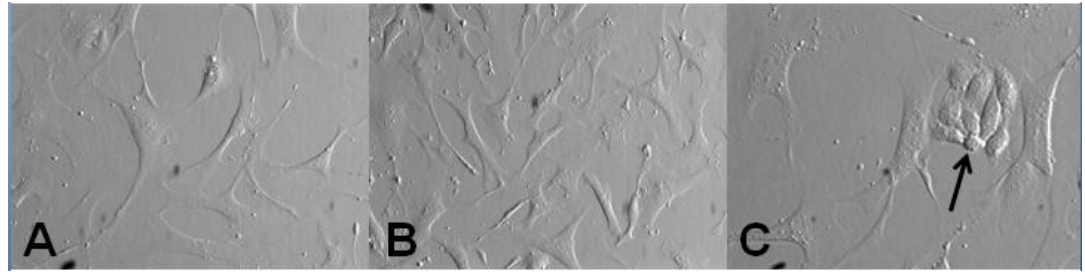


Figure 2-1 Primary cardiac fibroblast

Images of WT PCFs. A) and B) illustrates PCFs at X20 magnification and C) illustrates PCFs at X40 magnification with a cluster of contaminating endothelial cells (black arrow).

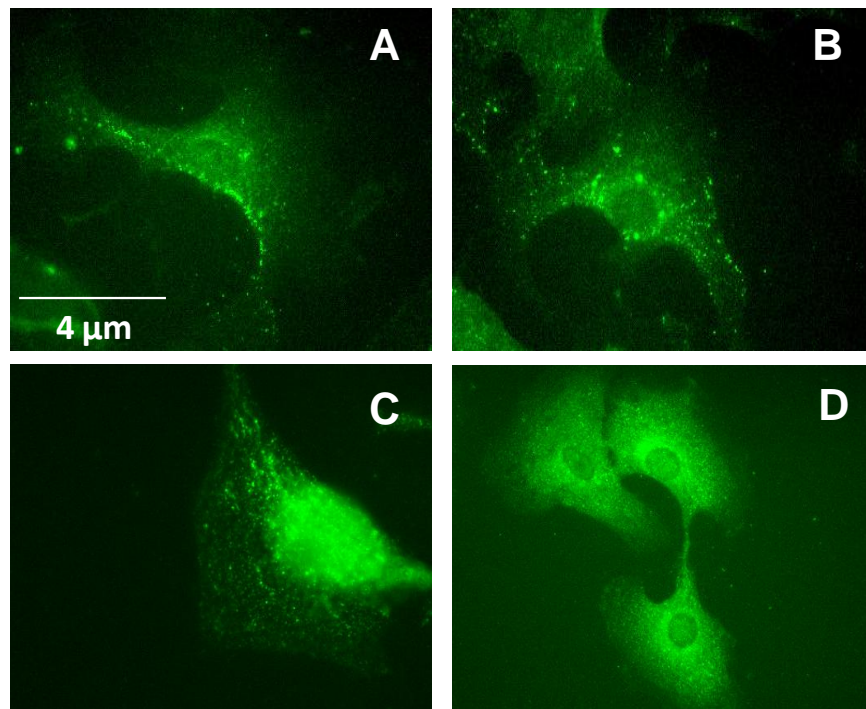


Figure 2-2 Immunocytochemistry staining

Immunofluorescence images (X40 magnification) for DDR2 in WT PCFs (A and B) and 3T3 fibroblast cell line (C) while (D) shows endothelial cells stained for von Willebrand factor.

2.3.1.2 FACS analysis

Flow cytometry was performed on WT PCFs passage 0 or 1 in order to quantify the purity of the cell isolation. The endothelial cells survive the collagenase digestion and comprise the main contaminating cell population in the initial harvested cell population. In order to quantify the percentage of endothelial cell contamination flow cytometry was undertaken for vWF (Figure 2.3 A & B). The gated M2 region in the non-specific TgG control was set to overlap with 5% of the non specific binding profile. (Figure 2.3 A) The M2 gated region in for the vWF FACS profile counted 10% of cells positive for vWF. (Figure 2.3 B) Therefore after subtraction of the 5% background of the non-specific IgG control resulted in a 5% contamination of the cardiac fibroblast with endothelial cells. However with passaging due to the numerically larger population of cardiac fibroblasts and their potential for vigorous growth they outgrow the contaminating endothelial cells (Table 2.3).

As no working antibody towards DDR2 was available for FACS this prevented the positive identification of the number of cardiac fibroblast in the initial cell isolation. Nevertheless an antibody against the surface antigen Cluster of Differentiation 90 (CD90) was available. In the heart this antigen is only expressed on fibroblasts. However the level of expression of CD90 varies between fibroblasts of different origin. Approximately 60-80% of orbital fibroblasts express CD90 (Tang et al., 2006). In contrast only 28% of fibroblasts from the lung of F344 rats and 15% in LEW rats are CD90 positive (McIntosh et al., 1994). Therefore it was anticipated that CD90 would not identify all the cardiac fibroblasts. (Figure 2.3 C) Quantification was performed as previously

with the M2 gate set at 5% for non-specific antibody binding. This was subtracted from the CD90 FACS value to give an average of 52%. Thus it appears in primary cardiac fibroblasts 52% of cells express CD90 and can be positively identified as cardiac fibroblast by FACS.

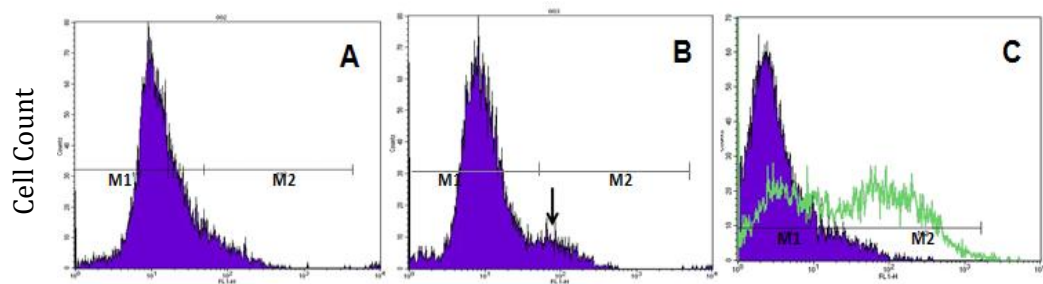


Figure 2-3 FACS profile of WT PCFs

FACS profile showing fluorescence (X axis: log scale) against counts (Y axis: cell number). (A) shows the IgG control with gates M1 set to catch 95% of the counts and gate M2 set to catch 5% of the counts. (B) illustrates an increase in the count in gated region M2 (black arrow). This was a 5% increase in the cell count for vWF expression. (C) illustrates a similar set up with M1 and M2 gated regions. The control non-specific IgG (purple) and CD90 (green) FACS profile are shown. There was a 52% increase in the M2 gated region in the CD90 FACS profile.

Table 2-4 Percentage of endothelial contamination assessed by FACS

The percentage of endothelial cell contamination decreases with passaging

Passage	Percentage endothelial cell contamination
Passage 0	5.05%
Passage 1	1.24%

2.3.2 HOMOGENATE: STIMULATION OF PCF WITH ANGII DID NOT INDUCE A ROS RESPONSE

The aim of the method was to measure the ROS production in homogenized PCFs after they had been stimulated with physiological concentrations of agonists. However, the intracellular ROS concentration of superoxide rarely exceeds 10 nM (Tarpey et al., 1999). As a result 10µg of protein was required to enable the measurement of basal levels of superoxide production.

No significant change in ROS production was observed with either aldosterone or AngII stimulation in both WT or Nox2^{-/-} PCF. (Figure 2.4) Similarly no increase in ROS was observed following TGFβ1 stimulation in WT PCF. The change in ROS production following stimulation with a range of different agonist has been quantified and tabulated in Table 2.4. Only AA stimulation resulted in a highly significant increase (p=0.002) in the ROS production in WT PCF (mean 3.901;95%CI 1.5-6.3 units) which was inhibited with DPI. This demonstrated that the WT homogenate was able to increase ROS production following agonist stimulation of a Nox isoform in a homogenate set up.

Under basal conditions, Tiron, the superoxide scavenger, significantly decreased ROS measurement in WT PCFs. (Figure 2.5) Similarly DPI, the flavoprotein inhibitor, caused a significant reduction in ROS production in both WT and Nox2^{-/-} PCFs (Nox2^{-/-} graph not shown). (Table 2.5) Together this suggests that our lucigenin assay was measuring superoxide and that NADPH oxidase was the main source of the basal ROS production in the WT PCFs. However the inhibition of ROS in the Nox2^{-/-} PCFs by DPI would suggested the possible presence of another Nox isoform as a contributing

source for the basal ROS levels. In contrast Rotanone and TTFA the mitochondrial site inhibitors, L-NAME the NOS inhibitor and allopurinol the xanthine oxidase inhibitor all caused a non significant reduction in the level of basal ROS production.

The main disadvantage of the homogenate technique was the large number of cells (300,000/60 cm² gave 3-4 triplicates of 10µg of protein) and therefore animals required for each experiment. As a result of this the adherent whole cell method was established with the aim of reducing the requirement for animals.

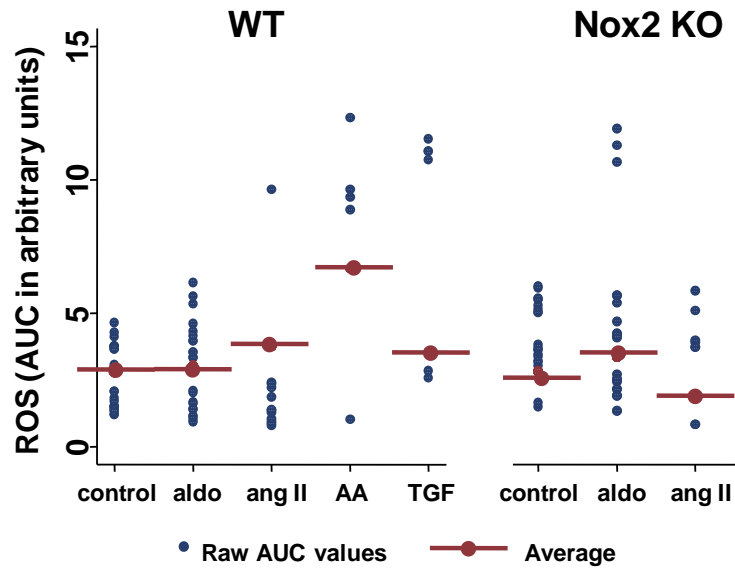


Figure 2-4 ROS production in WT and Nox2^{-/-} homogenates following stimulation with agonist

There was no difference in the baseline ROS production between WT and Nox2^{-/-} PCFs. Stimulation with arachidonic acid demonstrated a significant increase in ROS production. No significant increase was seen following stimulation with the other agonists.

Table 2-5 Change in ROS production in WT and Nox2^{-/-} homogenates following agonist stimulation

Stimulation	Difference in AUC from WT controls	p-value	95% Conf.Interval	
Aldo (10μM) WT <i>n</i> =7	0.259	0.457	-0.436	0.953
Ang (10μM) WT <i>n</i> =4	0.968	0.109	-0.226	2.162
AA (30μM) WT <i>n</i> =2	3.901	0.002	1.508	6.294
TGF (10ng/ml) WT <i>n</i> =2	0.757	0.637	-2.419	3.932
	Difference in AUC from Nox2 ^{-/-} controls			
Aldo (10μM) NoxKO <i>n</i> =7	0.551	0.598	-1.540	2.642
Ang (10μM) NoxKO <i>n</i> =3	-0.926	0.470	-3.488	2.642

Control: WT n=8 NoxKO n=7

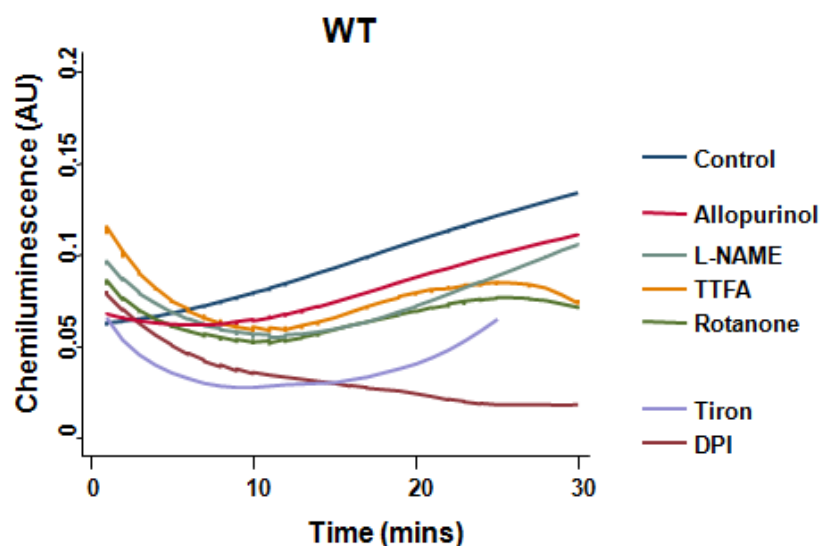


Figure 2-5 Average pharmacological inhibitor profile of ROS production in WT PCF homogenate.

All the inhibitors resulted in a reduction in basal ROS production. Statistically significant inhibition was seen only with DPI and Tiron. Similar profile was observed with Nox2^{-/-} PCFs.

Table 2-6 Reduction in ROS production following pharmacological inhibition in WT and Nox2^{-/-} homogenate

Inhibitors	Difference in AUC from control in WT	p-value	95% Conf.Interval	
DPI <i>n</i> =4	-1.682	0.003	-2.782	-0.582
Rotenone <i>n</i> =7	-0.486	0.120	-1.101	0.129
TTFA <i>n</i> =6	-0.318	0.204	-0.811	0.175
L-Name <i>n</i> =6	-0.250	0.305	-0.732	0.231
Allopurinol <i>n</i> =5	-0.059	0.818	-0.570	0.451
Tiron <i>n</i> =3	-1.465	<0.001	-1.951	-0.980
	Difference in AUC from control in Nox2 ^{-/-}			
DPI <i>n</i> =2	-1.47	<0.001	-2.15	-0.80
Tiron <i>n</i> =2	-1.16	<0.001	-1.35	-0.96

2.3.3 ADHERENT WHOLE-CELL ROS MEASUREMENT

A significant disadvantage of the homogenate based method was that large numbers of cells were required to provide sufficient protein to assess baseline and stimulated ROS production. Since the adherent cell method allows the assessment of ROS in a whole-cell format, this ROS production may more truly reflect the balance between oxidation and antioxidative pathways and be more representative of the *in vivo* situation. Furthermore homogenising the cells may obscure a small stimulatory effect of aldosterone or angiotensin II by disrupting ROS localisation and cellular signalling pathways.

There is no known mechanism to transport NADPH into intact cells. Additionally, lucigenin, which is a large molecule of 510.1 daltons in size, has been reported to react exclusively with extracellular ROS production in intact neutrophils (Dahlgren et al., 1985). Consequently, to ensure the unrestricted access of NADPH to the inside of the cells saponin, a mild detergent that creates pores in cholesterol containing membranes, was used to permeabilise the cells (Francis et al., 2002). A previous study has reported that cells permeabilised with 0.05% saponin showed no loss of cell viability (Endo and Iino, 1980).

2.3.3.1 Surface coating influenced basal ROS production

Extracellular matrix (ECM) has been shown to stimulate ROS production. Fibronectin and laminin stimulated ROS production by activation of Nox4 through products of the phospholipaseA2 (PLA2)/lipoxygenase (LOX) pathway (Edderkaoui et al., 2005). Therefore

the change in basal ROS production in WT PCF was investigated. Cells were added to 96 well plates which were pre-coated with fibronectin, laminin, collagen and gelatin for 16 hrs. Laminin, fibronectin and collagen are commonly used in our laboratory for coating cell culture plates to encourage cell adherence.

PCF seeded onto any of the coated surfaces had a significantly higher basal ROS production as compared to cells on an uncoated surface. (Figure 2.6) However this increase may simply be the result of increased number of cell adherence to the plate. Since the basal ROS levels were not significantly different between the different coated surfaces (Table 2.6) and as gelatin was regularly used for coating plates in our laboratory, gelatin was used for future experiments.

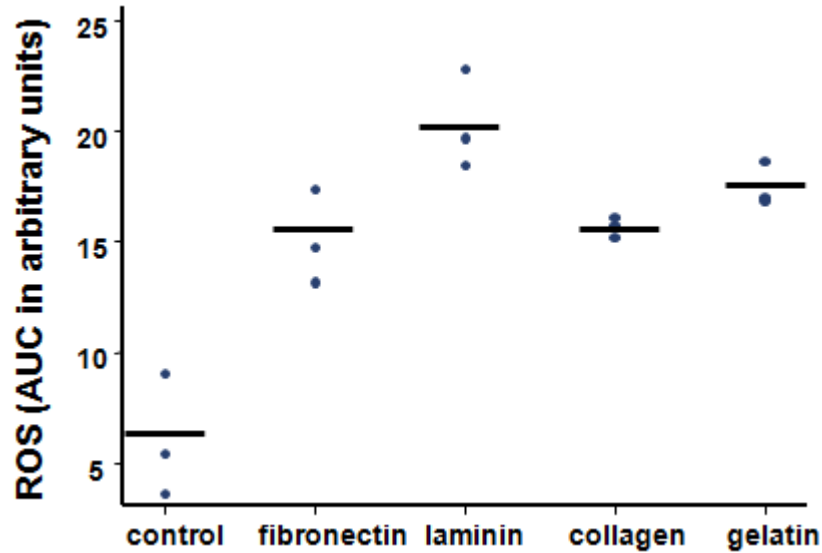


Figure 2-6 Basal ROS production in WT PCF in response to different surface coatings.

The wells were pre coated for 16hrs with either 25ng/ml of fibronectin, 25ng/ml laminin or 10ng/ml of gelatin or collagen. Excess coating was removed and the PCFs added to the wells. Basal ROS levels were compared to control well which were left uncoated.

Table 2-7 Comparison of the change in basal ROS in WT PCFs on different surface coatings.

	Average baseline ROS values	Difference from gelatin coating	p-value	95% Conf.Interval	
Gelatin (<i>n</i> =3)	17.487	-	-	-	-
un-coated (<i>n</i> =3)	6.044	-9.457	<0.001	-11.426	-7.489
Fibronectin (<i>n</i> =3)	15.086	-2.059	0.143	-4.880	0.763
Laminin (<i>n</i> =3)	20.303	1.054	0.328	-1.145	3.253
Collagen (<i>n</i> =3)	15.668	-2.052	0.039	-3.986	-0.119

2.3.3.2 Assessment of the effect of washing on ROS production

After permeabilisation of the adherent cells with saponin constituents present in the medium can enter the cell and scavenge the ROS such as BSA. Also pyruvate is present in the cell culture media, which can bolster the endogenous glutathione antioxidant system and thus reduces ROS (Mallet, 2000). Similarly it has been reported that the pyruvate in myocardial homogenates, dose dependently, reduced ROS production. However incorporation of a wash step to remove all traces of the culture media could lead to cellular detachment. To assess the effect of these factors, baseline ROS production of WT and Nox2^{-/-} cells seeded onto gelatine coated 96 well plates was assessed in media, after removal and replacement of medium with 75µL IC solution and after the removal of media, washing with PBS and replacement with 75µL IC solution.

The results demonstrate that ROS levels both in WT and Nox2^{-/-} were significantly ($p < 0.001$) lower when measured in medium as compared to IC solution. (Figure 2.7 & Table 2.7) Inclusion of a further PBS wash step had no significant effect on the basal ROS measurements. In order to eliminate the possible quenching effect of medium on ROS production in all future experiments media was removed, cells washed with PBS and IC solution added.

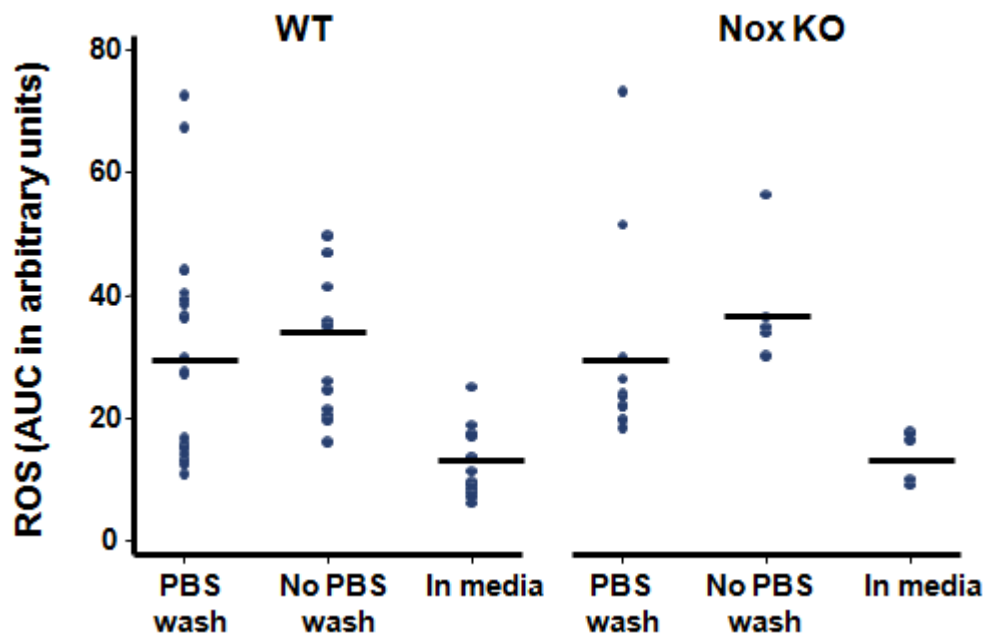


Figure 2-7 ROS levels in WT and Nox2^{-/-} cells with different wash protocols.

Basal ROS levels were measured in WT and Nox2^{-/-} in media or media replaced with IC solution or media removed, 96 well washed with PBS and replaced with IC solution. ROS measurement was significantly lower in medium as compared to IC solution. The additional wash step did not significantly alter the basal ROS measurement.

Table 2-8 Comparison of ROS levels with different wash protocols.

	Mouse adjusted average baseline ROS production	SEM	Difference from PBS wash	p-value	95% Conf.Interval	
PBS wash	30.519	2.329	-	-	-	-
no PBS wash	34.835	3.199	4.316	0.280	-3.588	12.219
media	13.206	3.200	-17.312	<0.001	-25.216	-9.409

2.3.4 WHOLE CELL ADHERENT: STIMULATION OF PCF WITH ANGII DID NOT INDUCE A ROS RESPONSE

Baseline ROS levels in whole adherent Nox2^{-/-} PCFs were significantly higher than that measured in WT. (Table 2.8 & 2.9) The effect of aldosterone and AngII at a range of different concentrations was examined in both WT and Nox2^{-/-} PCFs. The dose response curves were performed at concentrations of 10⁻⁹ M to 10⁻⁶ M for both aldosterone and AngII. No stimulatory ROS effect of either aldosterone or AngII was seen at any of the concentrations used. (Table 2.8) Arachidonic acid was used as a positive control and the response to the classical profibrotic agonist TGF was also assessed. AA significantly increased the ROS levels in both WT and Nox2^{-/-} PCF by a similar amount. (Table 2.8 & 2.9) However TGFβ only induced a significant increase in ROS in WT PCFs. (Table 2.8 & 2.9)

Table 2-9 Average ROS levels adjusted for runs measured in adherent WT and Nox2^{-/-} PCF

	WT			Nox2 ^{-/-}		
	Average ROS levels	SEM	N	Average ROS levels	SEM	N
Unstimulated	30.787	1.512	8	48.115	3.181	4
AA (30μM)	57.033	3.747	7	75.303	4.521	3
TGFβ (10ng/ml)	35.388	1.272	7	50.550	3.606	3
Aldosterone 10 ⁻⁹ M	34.008	2.104	3	-	-	-
Aldosterone 10 ⁻⁸ M	28.253	2.767	3	-	-	-
Aldosterone 10 ⁻⁷ M	29.160	2.258	3	-	-	-
Aldosterone 10 ⁻⁶ M	30.250	1.939	8	48.048	4.352	4
Angiotensin II 10 ⁻⁹ M	30.840	3.337	3	-	-	-
Angiotensin II 10 ⁻⁸ M	29.148	2.140	3	-	-	-
Angiotensin II 10 ⁻⁷ M	34.701	1.609	8	49.048	3.587	4
Angiotensin II 10 ⁻⁶ M	30.967	2.042	3	-	-	-

Table 2-10 Summary of ROS production in WT and Nox2^{-/-} following stimulation with AA or TGFβ

Comparisons	Difference	p-value	95% Conf.Interval	
Baseline ROS levels WT vs NoxKO	17.329	<0.001	9.017	25.640
ROS stimulation by AA in WT	26.246	<0.001	18.338	34.154
ROS stimulation by AA in NoxKO	27.188	<0.001	20.381	33.995
ROS stimulation by TGF in WT	4.601	0.003	1.600	7.602
ROS stimulation by TGF in NoxKO	2.435	0.236	-1.628	6.497

2.3.4.1 Inhibition of ROS production in adherent WT and Nox2^{-/-} PCF showed a similar source of basal ROS production

A range of pharmacological inhibitors were used to investigate the source of ROS. NADPH is a substrate for a number of different enzymes and the use of pharmacological inhibitors helps to define the percentage of ROS production attributable to NADPH oxidase. As there was no stimulatory increase in ROS production with aldosterone or angiotensin II only the difference in basal ROS production between WT and Nox2^{-/-} PCFs was investigated.

The inhibition profiles looked similar in both WT and Nox2^{-/-} PCFs in that DPI and Tiron completely eliminated the ROS production whereas rotenone only partially decreased it. (Figure 2.8) This partial reduction in ROS production by rotenone was not observed in the homogenate method. Statistical analysis demonstrated that DPI, rotenone and Tiron all decreased the basal ROS level significantly ($p < 0.001$). However further comparison between DPI and rotenone demonstrated that DPI decreased the basal ROS level significantly more than rotenone ($p < 0.001$) This suggests that mitochondrial ROS contributed 51% of the measured ROS production and NADPH oxidase contributed the remaining 46%.

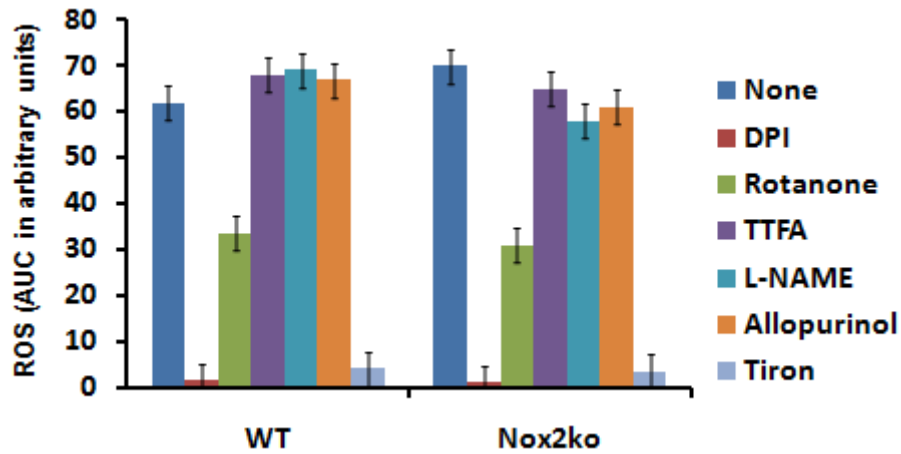


Figure 2-8 Inhibitor profile of baseline ROS production in adherent WT and Nox2^{-/-} PCFs

PCFs (5000 cells/well) were incubated for 24hrs. Inhibitors were added 5 minutes prior to measurement. DPI (10 μ M) and Tiron (5mM) abolished all ROS production. Rotenone (10 μ M) demonstrated a partial reduction in the ROS level. This pattern was observed in WT and Nox2^{-/-} PCFs.

2.4 DISCUSSION

AngII stimulation of rat cardiac fibroblasts has been shown to increase collagen production which is required for the development of fibrosis (Lijnen et al., 2001). The lack of fibrosis following AngII stimulation in Nox2^{-/-} mice suggested that the process was redox sensitive and in particular Nox2 dependent. Therefore the ROS response in cardiac fibroblasts, and in particular that from Nox2, to AngII stimulation was investigated in this chapter.

2.4.1 CELL CULTURE AND ROS MEASUREMENT

A cell culture model for adult mouse cardiac fibroblasts was developed to investigate the ROS response to AngII stimulation. Although the use of a cell culture approach isolates the PCFs from their local environment in the heart it does enable the investigation of the response in that particular cell in isolation. Most studies in the literature have been carried out in neonatal or adult rat cardiac fibroblasts. The isolation of PCFs from adult mouse heart by collagenase digestion is a very sensitive process. Initial isolation attempts were unsuccessful with Worthington's collagenase type 2 despite using identical protocols. A change to Liberase Blendzyme IV Roche, which is a mixture of collagenase type 1 and 2, proved successful.

Fibroblasts were identified by their spindle shaped morphology and DDR2 staining. Cardiomyocytes are destroyed by the digestion process and therefore endothelial cells are the main contaminating cells. Approximately 5% of cells were positively identified by FACS analysis as endothelial cells using vWF as an endothelial cell marker. This percentage of cells decreased

after first passage to approximately 1% resulting in a relatively pure cell population for experimentation.

The ROS measurement was undertaken by two methods namely homogenate and whole cell adherent methods. The homogenate method is a well established method used by a number of research groups, including our own laboratory, to detect ROS production from whole heart or aortic homogenates (Belaiba et al., 2007; Byrne et al., 2003; Johar et al., 2006). In the homogenate method the intracellular signaling pathways and negative feedback loops are disturbed and hence the NADPH oxidase complex is unopposed by any restraining factors, such as SOD, and can produce superoxide freely. Also the lucigenin and NADPH substrate have uninhibited access to the superoxide and superoxide producing components. However the significant disadvantage of this process was the large number of cells, approximately 250000 cells, required to achieve 10 µg of protein.

The whole cell adherent method was less demanding on cell numbers (5000cells/well) and would therefore enable more replication of experiments and investigations with agonists and inhibitors. The whole cell adherent method may be more representative of the *in-vivo* situation where the whole cell has its oxidative and antioxidative pathways present and in spatial proximity. Hence the measured activity in the whole cell adherent method is more likely to reflect the balance between the oxidative and antioxidant pathways. Adherence of the PCFs to collagen, laminin or fibronectin increased baseline ROS production in each by a similar amount. ROS production following cell receptor and ECM protein interaction is well known and is probably the cause for this increase in ROS (Borgquist et al., 2002). Whilst the presence of constituents in cell culture media such as pyruvate

and BSA, which scavenge ROS, significantly reduced the ROS measurement. Hence the whole cell adherent method was standardized by all experiments being undertaken on gelatin coated plates and the ROS measured after the culture medium was removed, cells washed and placed in IC solution.

2.4.2 *ROS RESPONSE*

MAIN FINDING:

- Basal ROS production in PCFs originates from NADPH and the mitochondria.
- Higher expression of Nox4 results in higher basal ROS levels in Nox2^{-/-} in PCFs.
- TGFβ stimulation of ROS production in PCFs is Nox2 dependent.

Despite the different protocols the results from both the homogenate and whole-cell adherent approaches were consistent. Arachidonic acid in both methods induced a significant ROS response which was not Nox2 dependent. Both WT and Nox2^{-/-} responded robustly suggesting the fibroblasts were able to increase their ROS production in response to an agonist and that the adherent methodology could detect such increases in ROS. It is known that AA stimulates Nox4 by activating the p22^{phox} subunit and also that the ROS production by AA stimulation is significantly reduced in cardiac fibroblasts transfected with Nox4 siRNA. Taken together these findings would suggest that the ROS production from AA resulted from the stimulation of Nox4 following the activation of the p22^{phox} subunit (Colston et al., 2005).

The basal ROS levels were significantly higher in Nox2^{-/-} PCFs. This is consistent with higher levels of expression of Nox4 in Nox2^{-/-} hearts (Byrne et al., 2003). Indeed the expression of Nox4 in Nox2^{-/-} PCFs was also investigated (by fellow colleague) and reliably found to be higher. The Nox4 isoform is constitutively active and therefore their higher expression levels would explain the higher basal levels of ROS production in Nox2^{-/-} PCFs.

Inhibitor experiments suggested that most of the basal ROS is NADPH oxidase dependent though a significant proportion is generated from the mitochondria. Rotenone did not inhibit ROS in the homogenate method whereas in the adherent method rotenone inhibited ROS production by 50%. This would suggest that the mitochondrion plays a role in the ROS production in the whole cell which is not observed after homogenizing thus demonstrating the advantage of the adherent technique.

TGFβ₁ stimulation did not result in any significant increase in ROS production in either WT or Nox2^{-/-} PCFs in the homogenate method. However the numbers of repeats which were possible with this method were small making the interpretation of this result difficult. In the whole cell adherent method a significant stimulatory ROS effect was seen in WT PCFs following TGFβ₁ stimulation. Interestingly this was not seen in Nox2^{-/-} PCFs suggesting that the actions of TGFβ₁ in cardiac fibroblasts could be Nox2 dependent. Evidence in the literature supports NADPH oxidase mediated effects of TGFβ₁. For example TGFβ₁ stimulation of rat kidney fibroblasts resulted in the rapid production of ROS along with increased expressions of Nox4 and importantly Nox2 (Bondi et al., 2010).

In both the homogenate and whole cell adherent methods no ROS stimulation was seen in either WT or Nox2^{-/-} PCF following AngII or

aldosterone stimulation. This is in contrast to findings in other published papers. Pagano et al (Pagano et al., 1998) reported an increase in ROS in adventitial aortic fibroblasts following 3 hrs of AngII(10^{-9} M) stimulation. Similarly Griendling et al (Griendling et al., 1994) reported an increase in ROS following AngII(10^{-8} M) stimulation in vascular smooth muscle cells using a homogenate based method. The lack of response here would question whether the resident cardiac fibroblasts used in our experiments are the principle site of the Nox2 dependent redox signal modulation resulting in the loss of cardiac fibrosis seen in the Nox2^{-/-} mice following AngII stimulation.

2.4.3 CELLULAR SOURCE OF NOX2 AND TGF β SIGNALLING

The cell source of the Nox2-dependent reduction in cardiac fibrosis following AngII stimulation would require an alternative hypothesis. The possibility exists that the experimental methods undertaken were not sensitive to detect a small ROS response. Nevertheless an alternative explanation could be that the fibroblasts involved in the development of cardiac fibrosis are recruited from epithelial and endothelial cells in processes termed epithelial-mesenchymal transformation (EMT) or endothelial-mesenchymal transformation (EndoMT). Zeisberg et al (Zeisberg et al., 2007a) demonstrated in mouse models of pressure overload following aortic banding that cardiac fibrosis was associated with fibroblasts of endothelial origin. Thus the paper suggests that the endothelial cells, and in particular those that undergo EndoMT to fibroblasts, are important in the development of cardiac fibrosis and thus could be the crucial resident cell source for Nox2. EndoMT has been shown to be induced by TGF- β and Smad signalling pathway (Zeisberg et al., 2007a). As yet, neither NADPH

oxidase nor ROS have been implicated in EndoMT in the heart. However, TGF- β /Smad induced epithelial-mesenchymal transition in the kidney has been shown to be inhibited by antioxidants (Rhyu et al., 2005). In addition, Smad3 may be particularly relevant to AngII induced cardiac fibrosis and inflammation, as a recent study demonstrated that AngII was unable to induce cardiac fibrosis in Smad3 deficient mice (Lan et al., 2008). Alternatively the fibroblast could be recruited from circulating fibroblast-like cells called fibrocytes that are derived from bone-marrow stem cells (Brittan et al., 2002; Bucala et al., 1994; Direkze et al., 2003; Ebihara et al., 2006). Thus the Nox2 in the fibroblast cells from these alternative sources could be a critical cell source for the Nox2 in the development of AngII induced cardiac fibrosis.

It is known that adult cardiac fibroblasts do contribute to cardiac fibrosis by transforming to myofibroblasts and increasing the deposition of ECM (Sun et al., 2002a). In a hallmark paper, Schultz et al (Schultz et al., 2002) tested whether AngII would be able to induce cardiac hypertrophy and fibrosis in vivo in the absence of TGF β_1 gene. The lack of the TGF β_1 gene fully prevented the development of cardiac hypertrophy and dysfunction induced by subpressor doses of AngII that was observed in wild type mice. Thus, this study provided the first direct evidence that AngII induced cardiac hypertrophy is mediated by TGF β_1 and indicate that TGF β_1 acts downstream of AngII and promotes myocyte growth and fibrosis in the heart.

TGF β_1 signalling via Smad2/3 is important in the transformation of fibroblasts to myofibroblasts and Nox4 has been shown to be important in this signalling process (Cucoranu et al., 2005). On stimulation of human cardiac fibroblasts with TGF β_1 Nox4 mRNA expression was significantly

increased and ROS production significantly decreased by siRNA against Nox4. Also investigation into the profibrotic role of TGF β_1 on rat fibroblasts has shown significant increases in NADPH oxidase activity with increased expression of Nox2 and Nox4 (Bondi et al., 2010). The downstream activation of Nox2 by TGF β_1 is in keeping with the Nox2 dependant ROS response found following TGF β_1 in the current experiments. Thus as TGF β_1 acts downstream from AngII this indicated that the fibroblasts could still be an important cell source for Nox2 in the development of cardiac fibrosis in mice following AngII stimulation.

2.4.4 ALTERNATIVE CELLULAR SOURCE OF NOX2

Another possibility is that macrophages are known key players and that their activation and migration into cardiac tissue and production of TGF β are significant events in the development of AngII induced cardiac fibrosis (Tokuda et al., 2004). In keeping with this, the ACE activity in monocyte-derived macrophages from pre-hypertensive patients has been shown to be increased (Keidar et al., 2007). Also, in hypertensive rats, macrophages accumulate in the perivascular area, co-localising with fibroblasts, and promote the increased production of collagen (Sun et al., 2002b). Thus monocytes/macrophages could be another possible critical cellular source for Nox2 in AngII induced cardiac fibrosis. In the next chapter bone marrow-derived macrophages (BMM) are used to investigate the role of Nox2 in the ability of BMM to migrate as a measure of the cell's ability to chemotax and infiltrate tissue. The dependence on Nox2 in this process, and the lack of it in Nox2^{-/-} mice, could explain the elimination of fibrosis in vivo in Nox2^{-/-} mice to angiotensin II or aldosterone stimulation.

3 ROLE OF NOX2 IN THE CELLULAR MIGRATION OF BONE MARROW DERIVED MACROPHAGE

3.1 INTRODUCTION

Fibrosis involves the infiltration of a dense inflammatory infiltrate including importantly macrophages (Swynghedauw, 1999). Adhesion of circulating monocytes to the endothelium of the vessel wall and the extravasation of the cell into the heart tissue is an essential step in the development of cardiac fibrosis (Kuwahara et al., 2003). In keeping with this, the blockade of monocytes chemoattractive protein -1 (MCP-1) early in this process prevents myocardial fibrosis, suggesting that the migration of macrophages into the perivascular space during inflammation is a key event in triggering fibrosis (Kuwahara et al., 2004). This migration requires the cell to make changes in their shape which is mediated by cytoskeletal dynamics. Several of the steps in the migratory process have been found to be redox sensitive. However the role of redox signaling and more specifically ROS generation by Nox2, in macrophage migration is not well established. However NADPH oxidase is recognised to have specific subcellular localisation and it is thought that this localization enables NADPH to produce ROS in a localised space and modulate specific signalling pathways that mediate various cell functions, including cellular migration (Ikeda et al., 2005b). In this chapter, we investigate the role of Nox2 in the migration of primary bone marrow derived macrophages.

3.1.1 MACROPHAGE MIGRATION

Our knowledge of the molecular mechanism regulating macrophage migration has developed from studies in various cell types. The initial

response of a cell to a migration-promoting agent is to polarize and extend protrusions in the direction of migration (Allen et al., 1998). These protrusions can be large, broad lamellipodia or spike-like filopodia, supported by actin filaments . (Figure 3.1) These adhesions serve as traction sites for migration as the cell moves forward over them, and they are disassembled at the cell rear, allowing it to detach (Ridley, 2001a).

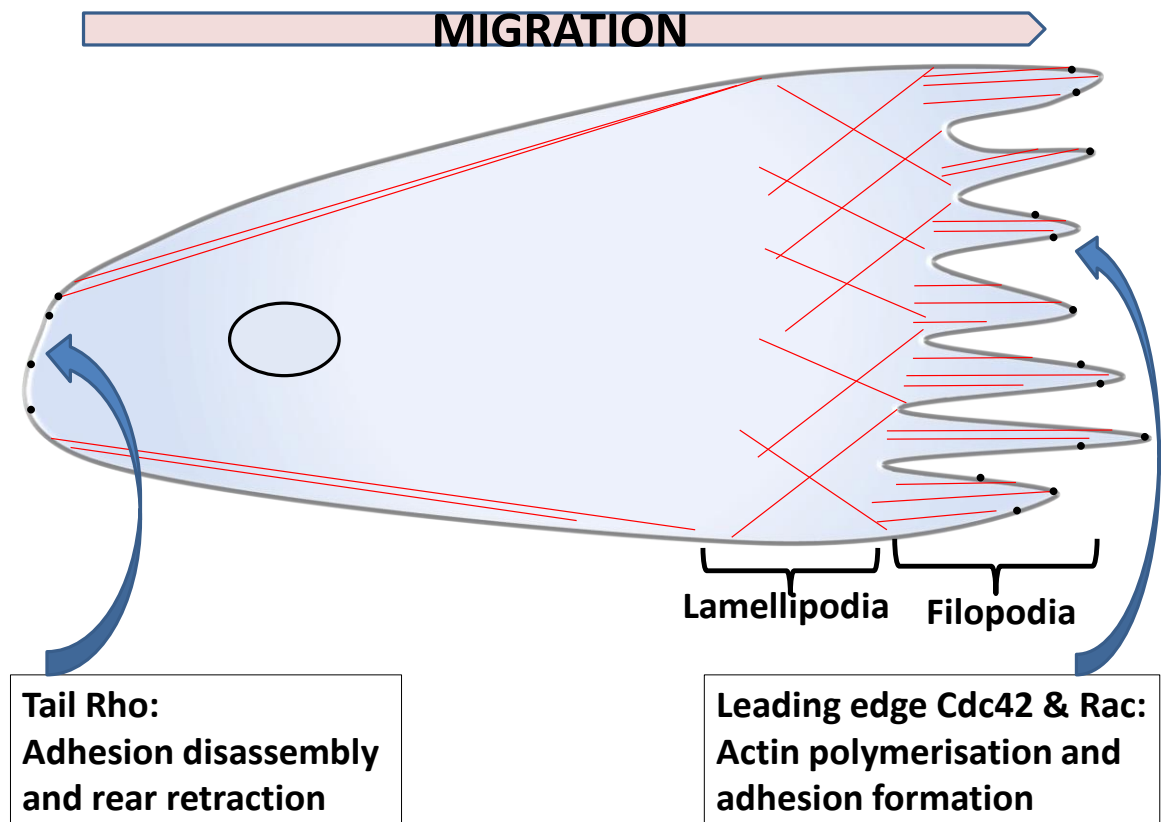


Figure 3-1 Migrating monocyte

The actin has a dendritic organization in lamellipodia. In contrast, filopodia, with their parallel bundle organization are particularly well designed to serve as sensors and to explore the local environment.

The Rho family of small GTPases(20-25kDa) are pivotal regulators of actin and adhesion organization.(Figure 3.2) Of the Rho family GTPases, Rac and Cdc42 are required for protrusion of lamellipodia and filopodia. Rac stimulates lamellipodial extension by activating WAVE proteins (Cory and Ridley, 2002). Cdc42 binds to WASP proteins, and in vitro this stimulates the Arp2/3 complex to induce dendritic actin polymerization and lamellipodial protrusion (Welch and Mullins, 2002). Another downstream signalling pathway for Rac and Cdc42 is their activation of the Ser/Thr kinase PAKs (p21-activated kinases). Upon activation PAK phosphorylates LIM kinase (LIMK) which phosphorylates and therefore inhibits cofilin. Cofilin is an actin binding protein involved in the assembly and disassembly of actin filaments. Hence its phosphorylation downstream from Rac and/or Cdc42 mediated activation is also involved in the regulation of lamellipodia formation.

Following directed stimulation, e.g in response to an inflammatory stimulus, for the cell to migrate it must become polarized. In order to do this the molecular processes at the front and the back of a moving cell are different. Again the small GTPases are important. Cdc42 is important in regulating cell polarity being active towards the front of migrating cells (Itoh et al., 2002). Its importance is confirmed by both inhibition and global activation of Cdc42 which disrupts the directionality of migration (Etienne-Manneville and Hall, 2002). Cdc42 can influence polarity by restricting where lamellipodia forms (Srinivasan et al., 2003).

Tail retraction is an active process involving RhoA. In several cell types inhibition of RhoA leads to the formation of an extended tail. This is possibly due to a decrease in the force generated by the interaction of actin

and myosin filaments, which is commonly termed the actomyosin based contractility (Worthylake et al., 2001). One model for how migrating cells maintain polarity is based on the fact that Rho and Rac are mutually antagonistic, each suppressing the other's activity (Evers et al., 2000).

The formation and turnover of cell-substratum adhesions is important to stabilise lamellopodia and for migration to occur. The integrins play an important part in such cell-substratum adhesions. The integrins are an important family of migration promoting receptors which connect the ECM to the intracellular cytoskeleton (Cox et al., 2001). Integrins can serve as both traction sites over which the cell moves and as mechanosensors, transmitting information about the physical state of the ECM into the cell and altering cytoskeletal dynamics (Geiger et al., 2001; Lauffenburger and Horwitz, 1996).

Activation of integrins results in them clustering and recruiting actin filaments. Actin is recruited to integrins by its cytoplasmic association via effector proteins such as talin, vinculin, α -actinin, filamin and paxillin. Both protein kinases and phosphatases appear to be central to the regulation of adhesion turnover and stability (Larsen et al., 2003). The focal adhesions organise the actomyosin apparatus and attract molecules such as focal adhesion kinases (FAK). Adhesion turnover in migrating cells is also regulated by the mitogen-activated protein kinase ERK (Brahmbhatt and Klemke, 2003; Turner et al., 2001). The emerging evidence favours a model for adhesion turnover in which activation of the protein tyrosine kinases FAK and Src accompanies the formation of an adhesion signaling complex that in turn mediates the localized activation of Rac and ERK. These signals then

contribute to the turnover of adhesions at the leading edge (Brahmbhatt and Klemke, 2003; Turner et al., 2001).

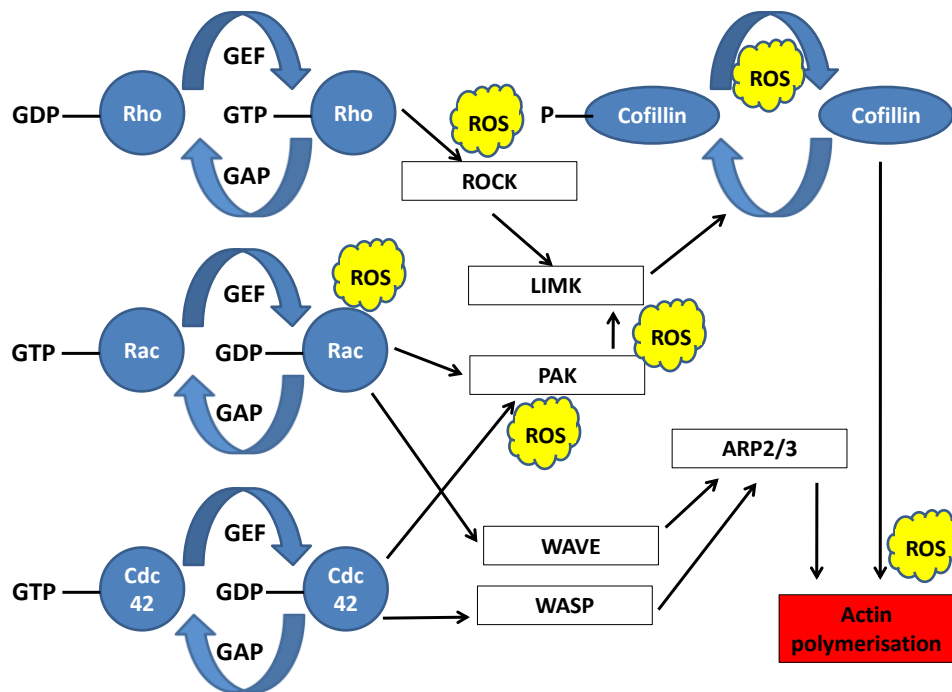


Figure 3-2 Regulation of actin polymerisation.

Rho, Rac and Cdc42 are activated by the exchange of Guanosine diphosphate (GDP) for Guanosine triphosphate (GTP) , catalysed by Guanine nucleotide exchange factors (GEFs). They are inactivated by the GTP hydrolysis, catalysed by GTPase activating proteins (GAPs). Cdc42 induces actin polymerisation by binding to WASP to induce branched actin filaments using ARP2/3 complex. Rac activates the ARP2/3 complex through the WAVE complex. Rac mediated or Cdc42 mediated activation of PAK (PAK1, PAK2 and PAK3) which phosphorylates LIMK (LIMK1 and LIMK2). Rho mediated activation of ROCK (ROCK1 and ROCK2) can also activate LIMK (LIMK1 and LIMK2). LIMK phosphorylates and therefore inhibits cofilin, thereby regulating actin filament turn over. Cofilin is dephosphorylated by the phosphatase Slingshot. Unphosphorylated cofilin stimulates severing and depolymerisation of filamentous actin. Redox sensitive pathways are indicated in as ROS.

In summary, a cell must sense a gradient and establish polarity. The plasma membrane is then extended in the direction of movement in the form of lamellipodia. Nascent adhesions are established in the front of the cell under the protrusion by restructuring of the actin cytoskeleton and changing its shape and size. Then a mechanical contraction force is induced by phosphorylation of myosin and the body of the cell contracts moving forward. Subsequently focal adhesions in the rear of the cell are detached and the trailing tail retracts. Finally the adhesion receptors are recycled by endocytosis and vesicular transport (Sheetz et al., 1998). These individual events are directed by activation of specific signals in the relevant subcellular compartment. Successful migration is thus dependent on many molecules, the activation and actions of which are carefully timed in pertinent subcellular compartments and this will determine the direction, speed and persistence of the migration. The remainder of this introduction discusses the role of ROS in regulating these steps in migration.

3.1.2 REDOX MODULATION IN CELLULAR MIGRATION

Several steps in the migratory process have been found to be redox sensitive. Signalling cascades activated by growth factors, such as PDGF, through their tyrosine kinase receptor require ROS to help propagate the signal further downstream (Lassegue et al., 1995; Lee et al., 2009). ROS can mediate integrin activation (Chiarugi and Fiaschi, 2007; Svineng et al., 2008) while focal adhesion sites have been reported to activate downstream ROS sensitive signal transduction pathways (Koller, 2002). These ROS can not only mediate cytoskeletal associated signal transduction by the oxidation of redox sensitive proteins such as protein tyrosine phosphatase (PTPs) but

also induce other pro-migratory growth factors (Black et al., 2008; Marmur et al., 1992; Pintucci et al., 2005).

Actin filament reorganisation is essential for cell size and shape changes, polarity formation and all phases of cell migration. Lamellipodia formation in moving cells requires cycles of actin polymerisation and depolymerisation. As shown in Figure 3.2. ROS can influence actin dynamics both directly and indirectly through alteration of intracellular signalling pathways. Treatment of actin with high concentrations of H_2O_2 has been shown to directly decrease the rate of polymerisation, elongation, increase monomer concentration and inhibit binding of actin capping protein, filamin (DalleDonne et al., 1995; DalleDonne et al., 1999; Milzani et al., 1997). The treatment of rabbit psoas muscle fibres with H_2O_2 results in decreased fibre contractility by the indirect impairment of the actomyosin enzyme activity (Price et al., 1998). However these studies used a high concentration of H_2O_2 which did not mimic the physiological state. In contrast lower concentrations of H_2O_2 seemed to promote actin polymerisation and the formation of stress fibres (Moldovan et al., 2000). However the effect of the oxidant on the actin cytoskeleton may be cell type specific. As such, Huot et al (Huot et al., 1997) showed that the same concentration of H_2O_2 induced fragmentation of F-actin in fibroblasts but reorganisation in endothelial cells leading to stress fibre formation. Figure 3.2 summarises the ROS dependent pathways leading to lamellipodium formation.

An important mechanism that regulates the contractile apparatus is the Rho/ROCK pathway which in VSMC has been shown to be activated by ROS (Jernigan et al., 2008; Jin et al., 2004). Several major downstream regulators of contractility from RhoA are ROS sensitive. For example the

regulation of Ca^{2+} release in VSMC is redox sensitive (Lounsbury et al., 2000; Roveri et al., 1992; Scherberich et al., 2000; Tabet et al., 2004) with Ca^{2+} dependent activation of MLCK being the major mechanism initiating cell contraction.

3.1.3 NOX MODULATION OF CELLULAR MIGRATION

There is now growing evidence in the literature that the Nox family proteins can also be a major source of ROS and thus have a modulatory effect on processes involved in cellular migration. For example, Rac stimulates actin polymerisation by several mechanisms including NADPH oxidase mediated ROS production (Moldovan et al., 2000). The dephosphorylation of the cytoskeletal regulator cofilin following PDGF stimulation has also been shown to be Nox1 dependent (Lee et al., 2009; San et al., 2008). During fibronectin/integrin mediated cell adhesion, ROS is dramatically increased by Rac-1 dependent activation of NADPH oxidase (Umanskiy et al., 2003). Other sources of integrin induced ROS include mitochondria (Werner and Werb, 2002) and lipoxygenase (Taddei et al., 2007). Recently Nox4 has also been shown to be a key player in the regulation of stress fiber formation and focal adhesion turnover in VSMC (Clempus et al., 2007).

Most of our understanding of the role of Nox2 in modulating cellular migration has come from studies in endothelial cells. Nox2 modulates cellular migration at various sites by means of specific subcellular localisation. (Figure 3.3) In endothelial cell migration, as seen during tissue repair in response to injury, angiogenesis, and wound healing, Rac1- and Nox2-dependent NADPH oxidase play an important role (Colavitti et al., 2002; Ikeda et al., 2005a; Ushio-Fukai et al., 2002). The Rac pathway is

involved in the production of ROS that accumulate at the membrane ruffles (Park et al., 2004), which is required for cytoskeletal reorganization and directed cell migration (Ikeda et al., 2005b; Moldovan et al., 2000). Nox2 and its regulatory subunits, p47^{phox} and p67^{phox} are also targeted to the focal complexes or membrane ruffles in lamellipodia (Ikeda et al., 2005a; Wu et al., 2003; Wu et al., 2005) thus enabling local activation of NADPH oxidase and ROS production.

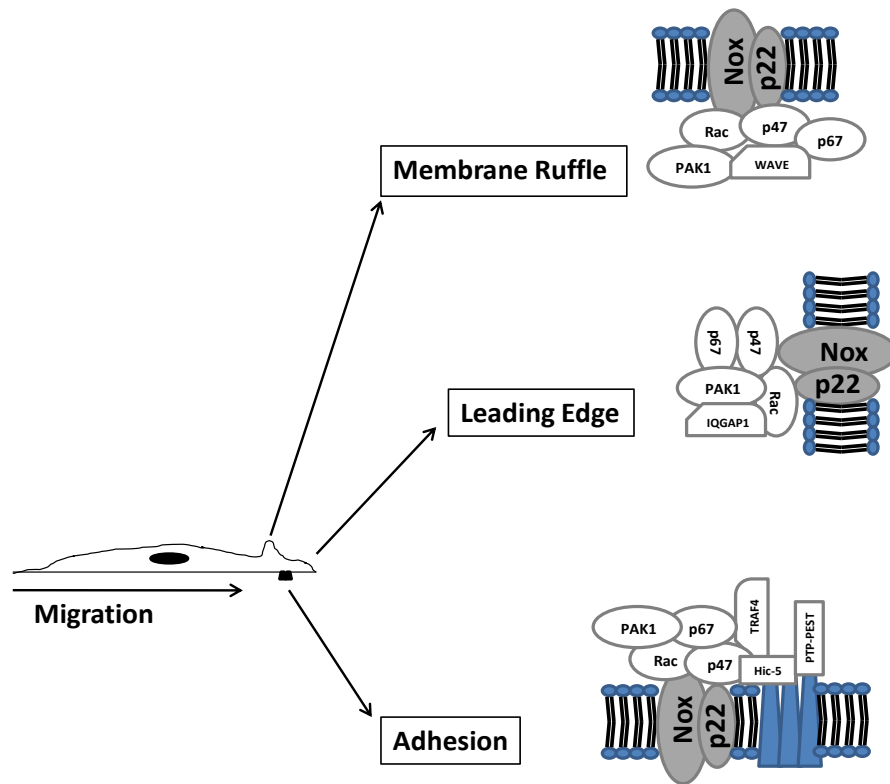


Figure 3-3 Localised ROS signalling.

ROS signalling at lamellipodial focal complexes is mediated through formation of p47^{phox}-TRAF4-Hic5 complexes and oxidative inactivation of PTP-PEST by ROS, which is required for activation of Rac1 and its effector PAK1, which phosphorylate p47^{phox} through formation of TRAF4-Rac1-PAK1 complexes. Localised ROS at membrane ruffles and lamellipodial leading edges are mediated through p47^{phox}-WAVE1-Rac1-PAK1 and Rac1-IQGAP1-Nox2 complexes respectively, which phosphorylate p47^{phox}. These NADPH oxidase targeting mechanisms allow for ROS dependent directional cell migration.

The mechanism for targeting NADPH oxidase to the lamellipodial leading edge and membrane ruffles is through the interaction of p47^{phox} with WAVE1 (Wientjes et al., 2001; Wu et al., 2003). The p47^{phox}-WAVE1 complexes contain Rac1 and Rac1 effector PAK1, which phosphorylates p47^{phox}. Antioxidants and inhibition of p47^{phox}-WAVE1 interaction block ROS production and ruffle formation (Wu et al., 2003). Another important targeting protein is IQGAP1 which functions as a scaffold protein to target Nox2 and Rac1 to the specific membrane compartments to localize ROS production, thereby achieving specificity of redox signaling, which may contribute to EC migration (Ikeda et al., 2005a).

3.1.4 SUMMARY

Cellular migration is a complex process which is tightly regulated. Many of these steps can be modulated by ROS. Compartmentalisation of NADPH oxidase, in particular Nox2, as a source of ROS allows for the modulation of specific signalling pathways. Most is known of the role of redox modulation in the migration of endothelial, fibroblast and VSM cells. The aim of the following studies was to specifically explore the role of Nox2 in the migration of BMM which have been identified as being important in initiating cardiac fibrosis (Sun et al., 2002b).

3.2 METHODS

3.2.1 *ANIMAL HUSBANDRY AND MAINTENANCE*

All the mice used in these experiments were maintained in a designated facility in accordance with the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures. Mice were housed up to a maximum of 5 per cage and had free access to water and normal food chow.

All experimental procedures in this thesis involving animals were carried out under the authority of a Home Office Personal Licence and Project Licence. All procedures were performed in strict accordance with the Home Office “Guidance on the operation of Animals (Scientific Procedures) Act 1986”, Her Majesty’s Stationary Office (London, UK).

3.2.2 *ISOLATION AND CULTURE OF MOUSE PRIMARY BMMS*

The murine femoral bones were harvested from 6-8 week old WT and Nox2^{-/-} mice after they were culled using terminal anaesthesia (2.5% isoflurane (Abbott, UK)//97.5% oxygen). All the surrounding tissue on the bone was meticulously removed using forceps in a tissue culture hood for sterility. Once the bone was cleaned a 21-gauge needle was used to pierce both ends of the bone. Using a 5-mL syringe the bone marrow was flushed out of the bone with 5 mL of macrophage starve medium (Table 3.1) into a 15-mL Falcon tube. An additional 5 mL of macrophage starve medium was added and the flush resuspended vigorously using a pipet. The cells were then centrifuged for 5mins at 1000rpm and the pellet resuspended in 5 mL of macrophage starve medium. The cells were then counted and 2×10^5 cells/cm² were seeded onto 10cm tissue culture plastic petri dishes for 3 days in macrophage growth media. (Table 3.1) Cells were thus harvested from

three WT and three Nox2^{-/-} mice with which all the experiments were conducted in chapter 3 and 4.

Table 3-1 Recipe for macrophage starve and growth media

Solution	Contents
Macrophage starve medium	RPMI 1640 with L-glutamine ADD 1% Essential amino acids 1% sodium pyruvate 1% P+S 10% heat inactivated FCS 0.5% βmercaptoethanol
Macrophage growth medium	This comprises of the starve media with the addition of M-CSF1 at 30ng/mL.

After 3 days the non-adherent population of cells containing the haematopoietic precursor cells were removed. The cells which attach to the bottom of the flask are a mixed population of differentiated hematopoietic cells and fibroblasts. The non adherent cells are centrifuged, resuspended and counted. The cells can then be cryogenically frozen in growth medium containing 10% DMSO at a cell density of 5×10^5 cells in each vial. To recover frozen cells the vials were rapidly thawed and 1 mL of warm growth medium was added dropwise. Then 4 further mLs of growth media was added and the cells centrifuged for 5 min at 1000g. The cells were seeded onto 6-cm bacterial culture plates in 5 mL of growth medium at a density of 10 cells/mL (i.e. a vial containing 5×10^5 cells seeded one 6-cm dish). The cells were

incubated for a further 5 days without refeeding. The cells in general remained in suspension for approximately 4 days at which point the differentiated BMMs became adherent and could be harvested on day 5 for experimentation.

To harvest the cells the medium was removed carefully and 2.5 mL of lysis solution such as Versene (Gibco) is added. The cells were incubated for approximately 20 min and then the cell suspension was collected by vigorous pipetting across the dish into a 15ml falcon tube. A further 2.5 mL of macrophage starve medium was added and the cells centrifuged for 5 min at 1000g. The cells were then resuspended in macrophage growth medium for seeding coverslips for staining or for Dunn chambers.

3.2.3 MIGRATION EXPERIMENTS

3.2.3.1 Random motion analysis

To study random cell motion, cells were seeded onto 6 well plastic petri dishes at a density of 2×10^4 cells/ml in macrophage growth medium and incubated overnight. Following incubation, cells were starved of CSF-1 in macrophage starve medium for 8 hours. The cells were then stimulated with CSF-1 by the re-introduction of CSF-1 (30 ng/ml) containing growth media. Cell images were collected using a Pulnix C CCD camera, taking a frame every 5 min for 18h using AQM acquisition software (Andor, UK).

3.2.3.2 Directed migration: Dunn Chemotaxis Chamber Preparation

The Dunn Chemotaxis Chambers (DCC) (Figure 3.4) were placed on tissue paper until the storage ethanol had evaporated. Initially the chamber was washed to remove any residual traces of ethanol; 100-150 μ L of starve medium was placed over the centre of the chamber, without overflow, and

then removed. This was repeated six times to wash the chambers. Then 150 μ L of starve medium was placed over the chamber until it filled both wells. The medium had been gassed by placing it in a bijoux tube in an incubator overnight in 5% CO₂, which was important as once the chamber is sealed by wax there is no opportunity for further gas exchange. Care was taken to invert the coverslip with the seeded cells attached onto the chamber without incorporating any bubbles into the chamber. The coverslip was placed slightly off centre so that a small gap remained in the outer well. However, the coverslip must completely cover the inner well so that the outer well could be drained without any loss of medium from the inner well. Once the coverslip was in place, the chamber was sealed on three sides with wax. The fourth side remained unsealed and the medium was removed from the outer well with Whatman paper. 100 μ L of growth media (with added chemoattractant) was added into the outer well through the gap, ensuring that it was bubble-free, until the well was full. The final edge was quickly waxed to completely seal the gap. The DCC was immediately placed onto the microscope stage and filming started.

3.2.3.3 Time-Lapse Microscopy and Migration Analysis

The assembled Dunn chamber was placed on the heated stage of a microscope. The live image from the microscope was then viewed on the computer monitor using the Acquisition manager (AQM) software (Kinetic Imaging; Liverpool, UK) and a region of the bridge was selected for recording and the image orientated such that the source of chemoattractant (outer well) was at the top of the screen. (Figure 3.4) This is extremely useful for subsequent tracking and mathematical analysis of chemotactic behaviour.

Ideally there should be 15-25 cells in the field of view and the cells should be evenly spaced. For analyzing the chemotactic behaviour of BMMs in a gradient of recombinant CSF-1, a time-lapse interval of 10 min was used and the cells filmed for 18h. CSF-1 is a known chemotactic factor which has been used in our laboratory before (Wells et al., 2004).

Once the random motion or directed motion assay were finished, the recorded sequence of images were analyzed using the same AQM software (Kinetic Imaging). Each cell was individually tracked (Figure 3.4) using the AQM software throughout the time-lapse sequence. In our laboratory the following tracking criteria has been adopted:

- Only cells present in the first frame were tracked.
- If any cell present in the first frame divided within the first 60 frames it was excluded.
- If a cell present in the first frame divided at a later stage in the film it was tracked until it ceased to be migratory. For BMMs this is normally one to two frames or 20 min before mitosis occurs.
- The daughter cells of this division were not tracked.

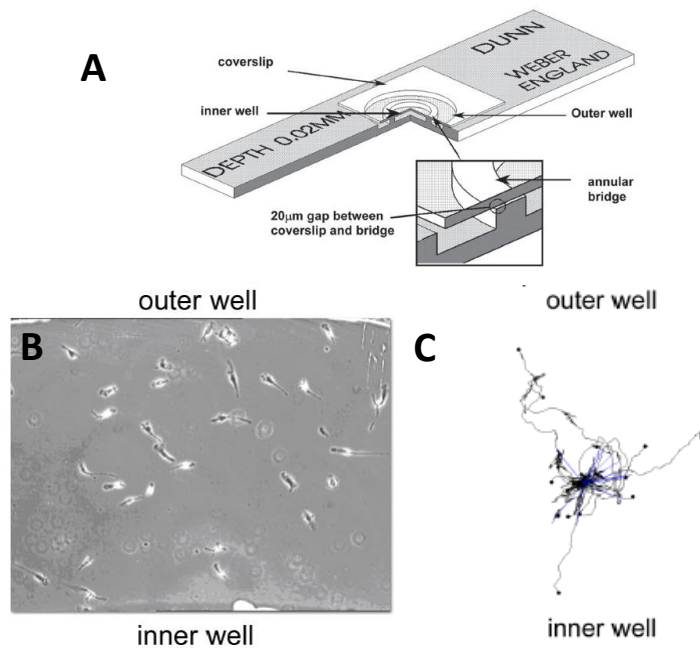


Figure 3-4 Dunn Chamber assembly, microscopy and tracking

A: The Dunn chemotaxis chamber with coverslip in place. The chamber consists of two concentric circles ground into one face of a glass slide (inner and outer wells). An annular ridge (bridge) separates the two wells. Insert, the bridge is 20 μm lower than the surrounding glass slide resulting in a gap between the coverslip and the bridge.

B: BMMs in the DCC as imaged by AQM Software. A region of the bridge is selected for recording and the image orientated such that the source of chemoattractant (outer well) is at the top of the screen.

C: Cell trajectories starting points are shifted to co-ordinate point (0,0) and plotted using an analysis program written in the laboratory on Mathematica software. (Zicha et al., 1997; Zicha et al., 1991)

Once all the appropriate cells in the field of view had been tracked the marked positions of each cell in each frame are saved. The files recorded the cell number, frame number, x-coordinate and y-coordinate of every tracked cell in every frame. Finally, trajectory analysis was conducted using a range of software designed by Dr. Graham Dunn and Dr. Daniel Zicha at the Randall centre, Kings College London (Zicha et al., 1991; Zicha et al., 1997) (software is not commercially available). This datasheet was transferred to Mathematica for statistical analysis. Chemotaxis analysis required the cells to reach a horizon defined in the analysis software package. Over the period of observation cells migrated towards this horizon up the positive CSF-1 gradient. Cells which reached the horizon were registered as having demonstrated positive chemotaxis.

In this analysis the assumption for the null hypothesis was that the cell trajectories have uniform distribution in all direction. The underlying distribution is one of a Rayleigh distribution. This program returns a Rayleigh test for unimodal clustering of direction. If the null hypothesis is rejected by the Rayleigh test the cells are showing a significant directional response to CSF-1.

3.2.4 *STAINING*

The BMM were seeded on the coverslips at 2×10^5 cells per coverslip. The cells were allowed to settle for one hour and then those for starvation and/or restimulation were starved of CSF-1 by replacement of the growth media with starve media for 12 hours. Following CSF-1 starvation the cells for restimulation with CSF-1 were stimulated by the reintroduction of the growth

media for 5mins. The cells were then washed with PBS and fixed with 1% paraformaldehyde for staining.

The cells on the coverslips were permeabilised with methanol and blocked with 5% BSA. The cells were stained for actin using phalloidin-FITC (1:1000) in 1% BSA. The staining was visualised under fluorescent microscopy and images taken. The image was analysed using ImageJ where the cells were traced to obtain their shape and size. Five random fields were chosen per image. The data from the staining were analysed using regression analysis where any clustering in the data arising from the fact that experiments were undertaken on the same run/day was allowed for and adjusted appropriately.

3.2.5 WESTERN BLOTTING

3.2.5.1 Protein samples

Cells were seeded onto 6-well plates. The media was not changed for growing cells while the medium of starved cells was replaced with macrophage starve media. Following 12 hours starvation the cells for CSF1 stimulation were stimulated for 5, 15, 30 or 60 mins by the re-introduction of growth media. After stimulation the wells were washed and lysis solution added. The 6 well plates were kept on ice. The wells were then scraped and the sample removed and placed into eppendorf tubes. The eppendorf tubes were spun for 10mins at 12000rpm and the supernatant removed into a new eppendorf tube. To denature the protein, samples were heated to 100°C for 3mins. Sample buffer was then added prior to freezing and storage.

3.2.5.2 Western blotting

To prepare 7.5% polyacrylamide gel the components included in Table 3.2 were added to a 50ml tube. Following the addition of TEMED (Sigma) which catalyzes the polymerization of acrylamide, the gel was poured into the Novex Mini-Cell Kit (Invitrogen) and left for 30 min to polymerize. The stacking gel solution (Table 3.2) was then poured over the separating gel. The well comb was placed immediately. The gel was left for 30 min to polymerize. Once the gel was set, the comb was removed. The gel was placed in the electrophoresis apparatus (BioRad). The gel tank was filled with 1x Running Buffer (all buffers and solutions applied in SDS PAGE are listed in Table 3.3). 10µg of each protein sample was loaded on the gel. 10µl of Dual Color Precision Plus Protein™ standard (Biorad) was co-electrophoresed with the sample. The gel was run at a constant 125V for 1.5 h.

Table 3-2 Recipe of 7.5% acrylamide gel

Solution	Contents	
7.5% Resolving Gel	H ₂ O	6.12ml
	3M Tris-HCl pH 8.8	1.25ml
	30% acrylamide (Biorad)	2.5ml
	10% SDS	100µl
	10% APS	100µl
	TEMED	10µl
Stacking Gel	H ₂ O	3.79ml
	1.5M Tris-HCl pH 6.8	0.65ml
	30% acrylamide	0.63ml
	10% SDS	50µl
	10% APS	50µl
	TEMED	10µl

3.2.5.3 Western Blotting protocol

The transfer cassettes, sponge pads, filter paper and the nitrocellulose membrane (Whatman) were equilibrated in 1x Gel Transfer Buffer (all buffers and solutions applied in Western Blotting Protocol are listed in Table 3.3). The Nitrocellulose membrane was placed on the gel. The transfer construct was assembled and placed in a transfer tank filled with 1x Gel Transfer Buffer. The transfer was run at 100V for 1h. Following the transfer, the membrane was blocked in blocking buffer for at least 1h at room temperature. The blocking buffer was discarded and replaced with primary antibody (included in Table 3.4) diluted with blocking buffer. The membrane was incubated with primary antibody overnight at 4°C on a rotary shaker. The next day, the membrane was washed three times for 20 min per wash in TBST, at room temperature. The membrane was then incubated with secondary antibody (Table 3.4) diluted with Blocking Buffer for 2h at room temperature. After incubation with secondary antibody, the membrane was washed three times for 20 min per wash, in TBST at room temperature. The blots were developed using Pierce® ECL Western Blotting Substrate Kit (Thermo Scientific) according to manufacturer's instruction. The equal parts of ECL detection reagents were mixed and immediately placed over the membrane. The membrane was incubated with this solution for 1 min at RT. The membrane was placed in a film cassette with a protein side facing up. The X-ray film (Fujifilm) was placed on the top of the membrane in a red safelight appropriate for X-ray exposure. The time of exposure was from 1-10 min depending upon the intensity of the signal. The blots were scanned and band densities were quantified with Kinetic Imaging software to obtain the ratio phosphorylated protein to total protein.

The data from the western blot analysis were analysed using regression analysis where any clustering in the data arising from the fact that experiments were undertaken on the same run/day or on the same gel was allowed for and adjusted appropriately.

Table 3-3 List of solutions used in Western Blot procedure

Solution	Contents
10x Running Buffer	<div> 144g glycine 30g Tris 10g SDS </div> } up to 1l H ₂ O
10x Gel Transfer Buffer	<div> 30g Tris 144g glycine </div> } up to 1l H ₂ O
Membrane Wash Buffer (TBST)	<div> 1M Tris-HCl pH 7.6 25ml 5M NaCl 10ml Tween20 1ml </div> } up to 1l H ₂ O
2x Gel Sample Buffer	100mM Tris/HCl pH 6.8 4% SDS 20 % Glycerol 0.2% Bromophenol Blue β-mercapto-ethanol ratio 1:50
Blocking Buffers	5% (wt/v) Skim Milk powder (Marvel) in 1x TBST 5% (wt/v) BSA powder (BDH) in 1x TBST(for experiment with phosphor-antibodies)

Table 3-4 List of primary and secondary antibody used in Western Blots

Primary Antibody	Dilution	Secondary antibody	Dilution	Antibody incubation Buffer
Akt	1:2000	Anti-rabbit secondary antibody	1:2000	1% milk powder in 1X TBS-Tween
phospho-p44/42 MAPK(Erk1/2) (Cell Signaling)	1:1000	Anti-mouse secondary antibody	1:2000	1% (wt/v) BSA powder in 1x TBS-Tween

3.3 RESULTS

3.3.1 MODULATION OF *Nox2*^{-/-} BMM CELL SHAPE

Many of the signalling pathways that regulate cellular migration are the same as those controlling cellular morphology. Therefore the WT and *Nox2*^{-/-} BMM cell morphology was analysed under basal growing conditions. Growing *Nox2*^{-/-} BMM had on average a larger spread area and were more elongated in their shape as compared to growing WT BMM. (Figure 3.5 & 3.6) On average growing *Nox2*^{-/-} BMM were 4% (0.03AU; p=0.048) more elongated with on average a 9% (0.022AU; p=0.05) increased spread area. Therefore these data suggests that *Nox2* plays a significant role in regulating the shape and size of BMMs.

3.3.2 ROCK INHIBITION DID NOT EMULATE THE *Nox2*^{-/-} PHENOTYPE

Rho Kinase (ROCK) has an important regulatory role in actin cytoskeletal dynamics (Bhadriraju et al., 2007). Furthermore ROCK is important for tail retraction (Smith et al., 2003; Worthylake et al., 2001), the inhibition of which can result in an elongated phenotype, as observed in *Nox2*^{-/-} BMM. Thus the influence of ROCK inhibition on WT shape changes was investigated.

The addition of a ROCK inhibitor significantly increased WT BMM spread area by 0.045AU (p=0.029). (Figure 3.5 & 3.6) (+19% compared with growing WT BMM). However inhibition of ROCK in WT BMM did not induce any significant elongation. Thus the complete phenotype seen in *Nox2*^{-/-} BMM could not be replicated simply by the inhibition of ROCK.

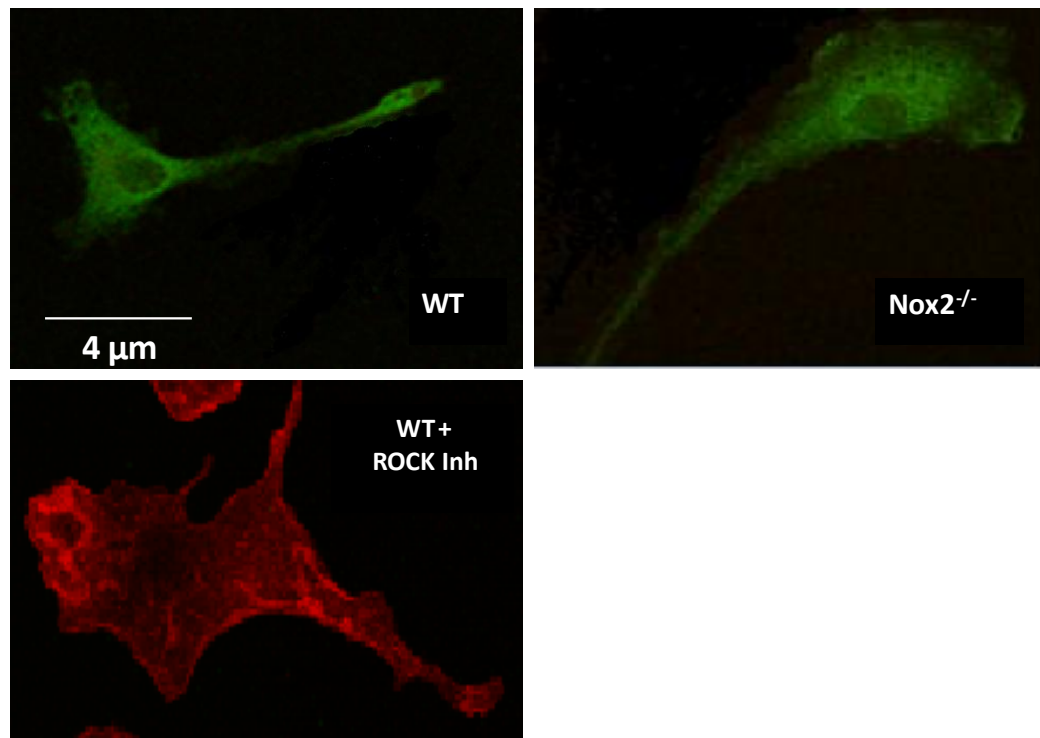


Figure 3-5 Differences in phenotype between WT, WT+ROCK inhibitor and Nox2^{-/-} BMM

The loss of Nox2 results in the BMM having on average a larger spread area and a more elongated shape. The addition of a ROCK inhibitor resulted in an increase in the spread area of the WT BMM. No change was seen in the elongation of WT BMM with ROCK inhibition.

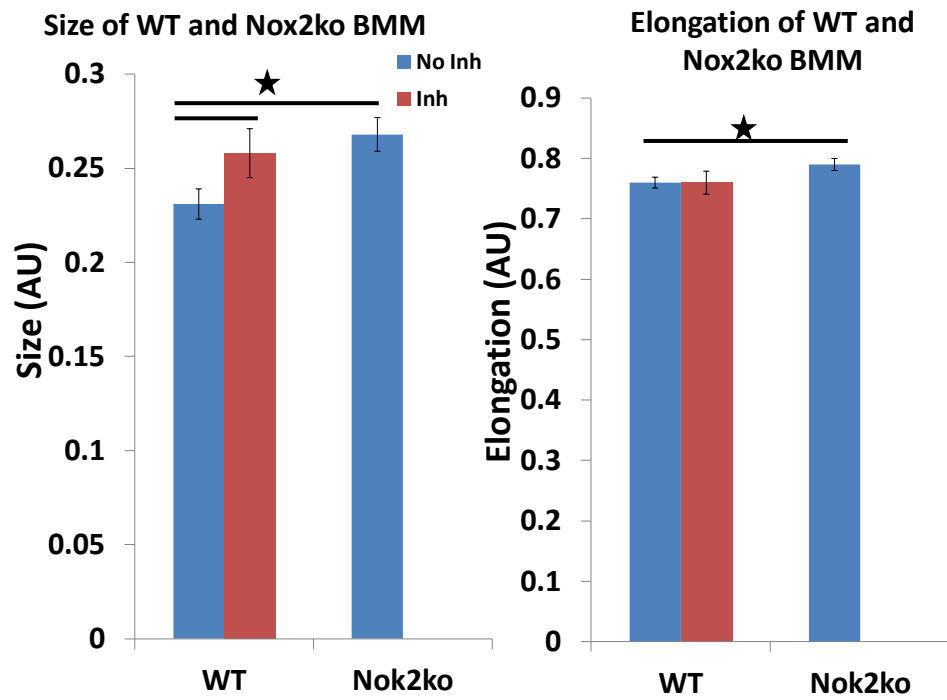


Figure 3-6 Area and shape changes in WT and Nox2^{-/-} BMM with and without ROCK inhibitor

Spread area and shape were quantified in arbitrary units (AU) using Image J. Loss of Nox2 resulted in an increase in the spread area and a significant increase in the elongation of the BMM. The addition of a ROCK inhibitor resulted in an increase in the spread area of WT BMM but no change was seen in cellular elongation. (★: $p < 0.05$, $N=150$ WT or Nox2^{-/-} cells)

3.3.3 CSF-1 STIMULATION IN WT AND NOX2^{-/-} BMM

BMM show a morphological response to CSF-1 stimulation (Wells et al., 2004) where deprivation of CSF-1 induces cell elongation and re-stimulation leads to centrifugal spreading. However the role of Nox2 in this response has not been investigated.

Following CSF-1 starvation both WT and Nox2^{-/-} macrophages reduced their spread area to a similar size which was not significantly different. (Figure 3.7) The re-introduction of CSF-1 allowed the assessment of the influence of Nox2 on the dynamic changes occurring in the cell's spread area and shape. Following the re-introduction of CSF-1 for 5mins the WT macrophages significantly increased their spread area by 79% (0.115 AU; $p < 0.001$) from its starting starved spread area. The Nox2^{-/-} macrophages also showed a significant increase in their spread area by 55% (0.087 AU; $p < 0.001$) from their starved spread area. This reduced response was not significantly ($p = 0.06$) different from the response seen in WT BMM.

After starvation WT and Nox2^{-/-} BMM had a similar degree of elongation. On stimulation with CSF-1 both cell types showed a similar reduction in their elongation as they spread out and became more rounded in shape. (Figure 3.7) The WT cells reduced their elongation by 11% (0.08AU; $p < 0.001$) similar to the 13% (0.095AU; $p < 0.001$) seen in Nox2^{-/-} BMM. Therefore both WT and Nox2^{-/-} BMM displayed a similar morphological response to CSF-1.

Although the difference in spread area was not statistically significant, taken together with the reduced cell area for growing WT cells, these data do suggest that Nox2 expression is in part required for normal BMM behaviour.

Interestingly, both populations were able to centrifugally spread in response to CSF-1, suggesting that CSF-1 responses are not completely dependent on Nox2 in BMMs.

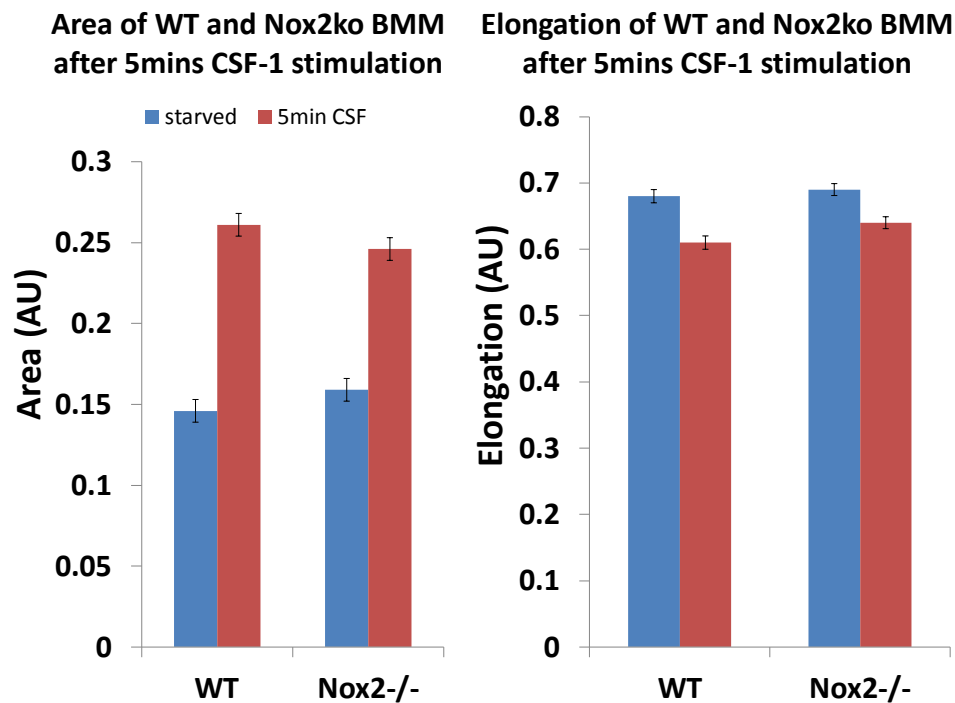


Figure 3-7 Shape and size changes to CSF1 stimulation in WT and Nox2^{-/-}

The increase in spread area is reduced in Nox2^{-/-} BMM however the decrease in elongation following CSF-1 stimulation is similar between WT and Nox2^{-/-} BMM. Taken together with the larger growing spread area of Nox2^{-/-} the data suggest a role for Nox2 in the modulation of downstream CSF-1 signaling resulting in differences in cell size.

3.3.4 Nox2^{-/-} BMM SHOWED REDUCED RANDOM MOTION FOLLOWING CSF-1 STIMULATION DO NOT MIGRATE TOWARDS CSF-1.

Given changes in growing cell spread area and a reduction in the spread area in response to CSF-1, defect in CSF-1 stimulated random migration in Nox2^{-/-} BMMs was investigated.

A significant reduction ($p=0.02$), in cell displacement was observed (Figure 3.8) following CSF-1 stimulation in Nox2^{-/-} BMM where a reduced number of the Nox2^{-/-} BMMs population were able to reach the set horizon as compared to WT BMMs.

The reduction in cell displacement could be the result of a reduction in cell speed or persistence in direction of motion. Interestingly the persistence in direction of Nox2^{-/-} BMM was higher than in WT. (Figure 3.8) However neither the persistence nor the speed were significantly different. On stimulation with CSF-1 a similar trend was observed. The speed and persistence increased in WT and Nox2^{-/-} BMM with a similar higher persistence in direction remaining in Nox2^{-/-} BMM, however no significant difference was observed.

Thus taken together the data suggests an important role for Nox2 in the random migration of BMM following CSF-1 stimulation. Despite similar speed of migration and a higher persistence in direction of motion the Nox2^{-/-} BMM were unable to randomly travel for longer distances to reach the set horizon.

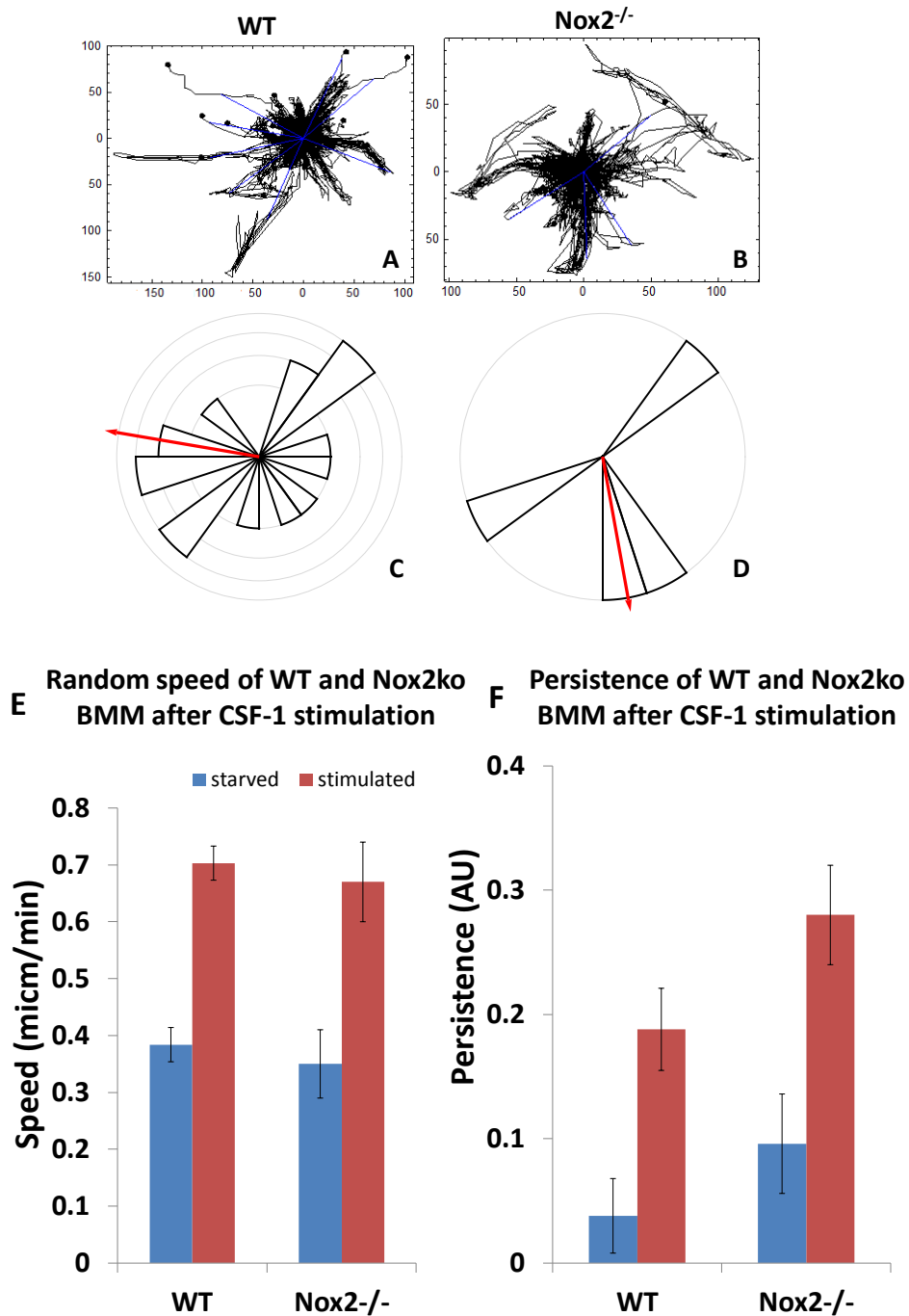


Figure 3-8 Nox2^{-/-} BMM have reduced cell displacement

WT and NOX2^{-/-} BMMs were seeded on plastic, CSF-1 deprived, then stimulated with CSF-1 and imaged (N=20 WT or Nox2^{-/-} tracks).

A & B: Cell tracks of WT and Nox2^{-/-} BMM respectively. The tracks have been re-set to co-ordinate (0,0).

C & D: The number of cells reaching a set circular horizon was monitored and found to be significantly ($p=0.02$) more in the WT than Nox2^{-/-} BMM following CSF-1 stimulation.

E & F: Mean cell migration speed and mean persistence of direction were calculated from cell tracks above. Persistence of direction was higher in Nox2^{-/-} BMM following CSF-1 stimulation. However no significant difference was observed.

3.3.5 Nox2^{-/-} BMM DO NOT MIGRATE TOWARDS CSF-1

BMM are known to have a chemotactic response to CSF-1 (Wells et al., 2004), and in a physiological context are likely to be responding to a gradient of chemoattractant rather than global stimulation. Thus the WT and Nox2^{-/-} BMMs were challenged to chemotax towards a source of CSF-1 using the Dunn Chemotaxis Chamber.

WT BMM demonstrated positive chemotaxis towards a CSF-1 gradient. (Figure 3.9) However the loss of Nox2 resulted in the loss of this positive chemotaxis towards the CSF-1 gradient, suggesting that Nox2 modulation of downstream signaling following CSF-1 stimulation is crucial in establishing directed migration.

3.3.6 Nox2^{-/-} BMMs HAVE REDUCED SPEED AND PERSISTENCE IN A CSF-1 GRADIENT

The loss of chemotaxis observed in Nox2^{-/-} populations could be accounted for by a reduction in cellular speed and/or reduction in the persistence of movement. Therefore the speed and persistence of the cellular motion was analysed.

The speed of migration of WT BMM towards a CSF-1 gradient was significantly greater than that seen in Nox2^{-/-} BMM ($p < 0.001$) (Figure 3.9). The persistence in the cellular migration towards the CSF-1 gradient was also significantly ($p < 0.001$) reduced in Nox2^{-/-} BMM as compared to WT (Figure 3.9). This suggests Nox2 plays an important role in the regulation of speed and the persistence in directed cellular migration of the BMM towards a CSF-1 gradient.

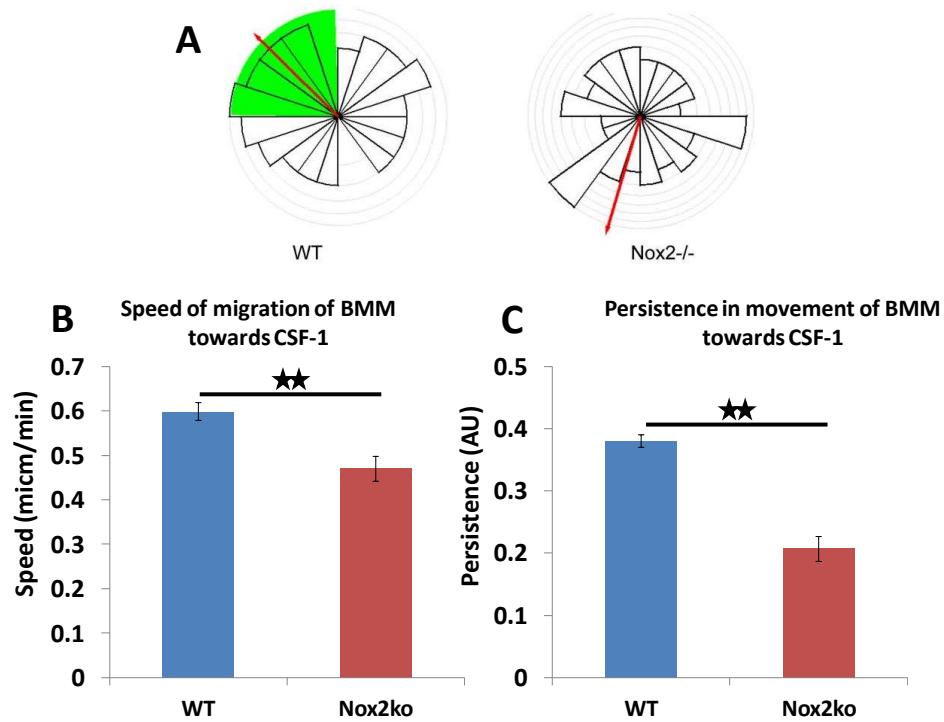


Figure 3-9 Loss of chemotaxis and reduction in speed and persistence of cellular motility following loss of Nox2

A: The loss of Nox2 resulted in a loss of chemotaxis towards a CSF-1 gradient. The WT cells showed an average upward movement (red arrow). This was significantly directed upwards as illustrated by the green wedged 95% confidence interval. Interestingly the average migration of the Nox2^{-/-} BMM was away from the CSF-1 gradient.

B & C: A significant reduction in speed and persistence of motion was seen in the Nox2^{-/-} BMM towards a CSF-1 gradient as compared to WT BMM. (★★: $p < 0.001$)

3.3.7 Nox2^{-/-} BMM SHOW REDUCED LEVEL OF ERK1/2 PHOSPHORYLATION FOLLOWING CSF-1 STIMULATION AS COMPARED TO WT BMM

Given the differences detected in both cellular morphology, cell spreading and directed cell migration, altered signalling downstream of CSF-1 in Nox2^{-/-} cells could explain this. In particular CSF-1 is well known to stimulate both ERK (Smith et al., 2008) and Akt phosphorylation (Sester et al., 2005) in BMMs and levels of ERK phosphorylation have been linked to cell spreading (Smith et al., 2008) and both ERK1/2 and Akt have an established role in cellular motility (Brahmbhatt and Klemke, 2003; Turner et al., 2001). Thus, this set of experiments investigated whether these signaling moieties are downstream of Nox2.

In response to CSF-1 stimulation both WT and Nox2^{-/-} macrophages showed a increased levels of ERK1 and ERK2 phosphorylation. (Figure 3.10) However the intensity of this response following 15mins of stimulation was significantly higher in the WT BMM for both ERK1 ($p < 0.001$) and ERK2 ($p = 0.01$) suggesting a role for Nox2 in ERK1 and ERK2 phosphorylation following CSF-1 stimulation. The duration of ERK1 and ERK2 phosphorylation was not significantly different between WT and Nox2^{-/-} BMM.

Akt phosphorylation, following CSF-1 stimulation, also increased in both WT and Nox2^{-/-} BMM by 15 minutes (Figure 3.10). However the level of phosphorylation at 15 mins and over the ensuing hour was not significantly higher in the WT BMM as compared to Nox2^{-/-} BMM ($p = 0.28$). Hence no Nox2 dependant effect was seen in the intensity or duration of Akt phosphorylation.

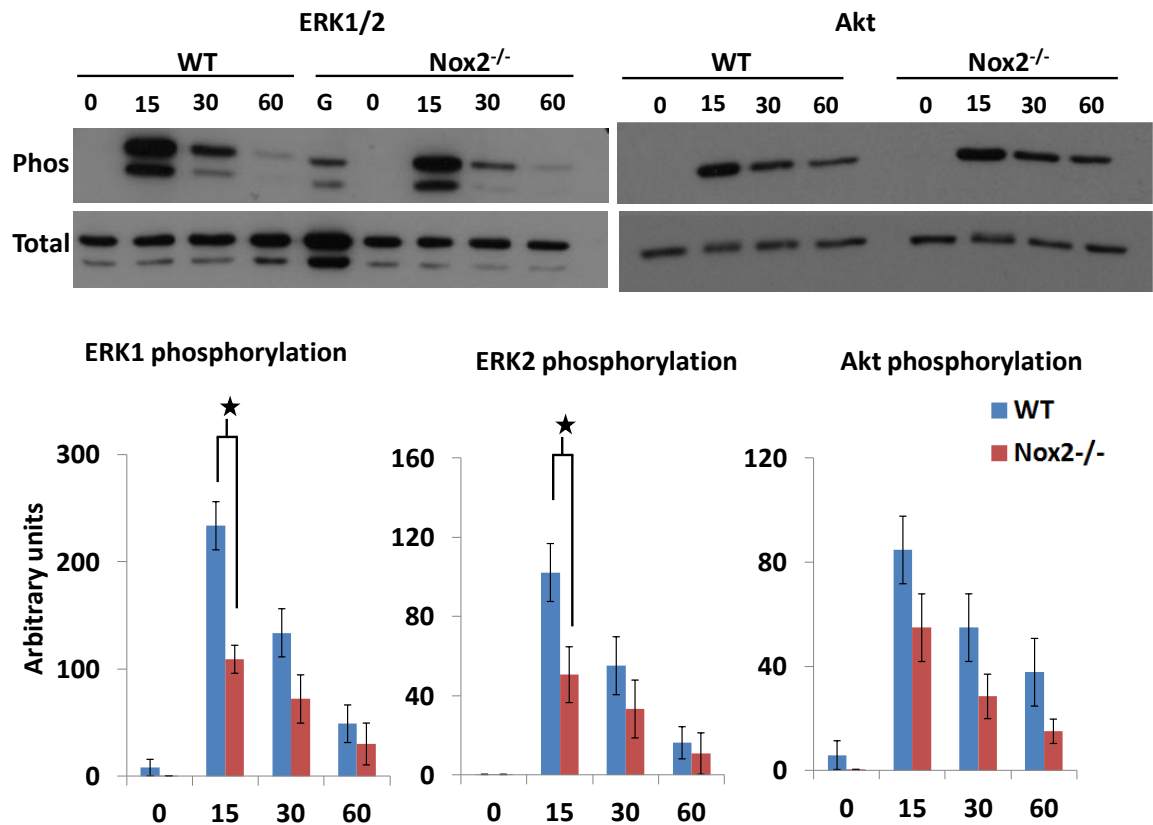


Figure 3-10 ERK1/2 and Akt phosphorylation in following CSF-1 stimulation

WT BMM had in general higher levels of phosphorylation than Nox2^{-/-} BMM following CSF-1 stimulation. This was significantly higher at time point 15mins (★:p<0.05) for ERK1 and ERK2. No significant difference was seen at any time point between WT and Nox2^{-/-} in their levels of Akt phosphorylation. (The phosphorylation blots were on the same gel and normalized against their total protein blots. Phosphorylation and Total protein blots are shown above.)(N=3)

3.4 DISCUSSION

The coordination and synergy between the cytoskeletal dynamics at the leading edge, the strengthening of adhesion to the ECM and cellular contractility play a key role in the dynamics of cellular morphology and migration (Ridley, 2001b; Ridley, 2001a; Ridley, 2004; Ridley, 2008). Redox signaling has been shown to be influential in this process at many different stages. In this chapter Nox2 has been shown to be important in regulating cellular morphology and to be critical in modulating cellular migration, speed and persistence.

3.4.1 *DUNN CHAMBERS*

Dunn chemotaxis chamber (DCC) slides were used to investigate the cellular motility. Chemotaxis can be studied using a Boyden chamber or transwell assay. However both these methods are based on scoring cells that have migrated into or through a filter membrane toward a source of putative chemotactic factor. In these assays, the local concentration gradients of chemotactic factor in and around the pores of the filter membrane are variable and unknown. Furthermore, the migratory behavior of the cells is unobservable and can only be deduced from the final distribution of the cell population. In contrast in the DCC, the migratory behavior of cells can be directly observed in a gradient of known direction and magnitude. The DCC allows the direct observation of slowly moving cells in a concentration gradient that is stable over longer periods of time (Dunn and Zicha, 1993; Zicha et al., 1991). The DCC chamber has been successfully used to characterize the migratory response of human macrophages to CSF-1 (Jones

et al., 2002), fibroblasts to platelet-derived growth factor (Zicha et al., 1991) and thrombospondin-1 (Orr et al., 2003), neutrophils to interleukin-8 (Zicha et al., 1998) and microglia to adenosine triphosphate/adenosine diphosphate (Honda et al., 2001). Hence the DCC in conjunction with time-lapse microscopy is a powerful tool that enables the user to observe directly the morphological response of cells to a chemoattractant in real time from which detailed information on the chemotaxis, speed and persistence of the cell migration can be obtained.

3.4.2 MIGRATION- DIRECTIONALITY

MAIN FINDING:

- Nox2 is crucial for chemotaxis towards a CSF-1 gradient.

A key finding was that the loss of Nox2 in BMM resulted in the complete loss of chemotaxis towards CSF-1. This indicated an important role of Nox2 in the ability of a cell to sense an external signal and/or in cellular polarisation.

Cells can respond directionally to very shallow chemoattractant gradients with a difference of less than 10% in the concentration of the chemoattractant between the front and rear of the cell (Devreotes and Janetopoulos, 2003). Such a small difference in signaling between the front and rear needs to be amplified into steeper intracellular signaling gradients in order to generate a cellular response. The phosphoinositides PtdIns(3,4,5)P₃ (PIP₃) and PtdIns(3,4)P₂[PI(3,4)P₂] along with PI3K and PTENS are key signaling molecules in this process (Merlot and Firtel, 2003;

Ward, 2004). This amplification process involves both localized accumulation and activation of PI3Ks, which generate $PIP_3/PI(3,4)P_2$, and the phosphatase PTEN, which removes them (Leslie et al., 2007). Cells with altered PI3K or PTEN activity can usually show chemokinesis but exhibit a significantly reduced chemotaxis (Procko and McColl, 2005; Ward, 2004). Many of these signaling molecules have been shown to be redox sensitive. Leslie et al (Leslie et al., 2003) demonstrated that oxidative stress with H_2O_2 resulted in the inactivation of PTEN. PTEN is a member of the Protein Tyrosine Phosphatase family which can be physiologically regulated through reversible oxidation resulting in their inactivation (Denu and Tanner, 1998; Lee et al., 1998). The inactivation of PTEN results in an increase in cellular phosphoinositides and thus the loss of any gradient established by the phosphoinositides to a chemoattractant signal. Moreover phosphoinositides, $PtdIns(3,4,5)P_3$ (PIP_3) and $PtdIns(3,4)P_2[PI(3,4)P_2]$, have been shown, by way of their Phox domains for subunits $p40^{phox}$ and $p47^{phox}$, to be involved in the recruitment and activation of these regulatory proteins, (Ellson et al., 2001; Ponting, 1996) thus establishing another means of redox modulation of the downstream signaling.

Following directed stimulation in order for the cell to migrate, it must become polarized. The small GTPases are important in this process and in particular Cdc42. Cdc42 is a master regulator of cell polarity by being active towards the front of migrating cells (Itoh et al., 2002) and by restricting where lamellipodia form (Srinivasan et al., 2003). Its importance is confirmed in experiments where both the inhibition and global activation of Cdc42 disrupts the directionality of migration (Allen et al., 1998; Etienne-Manneville and Hall, 2002). In contrast to this the loss of Rac did not affect

chemotaxis in BMM to CSF-1 (Wells et al., 2004). How Cdc42 and Nox2 are associated is not entirely clear however evidence from the literature suggest that in an *in-vitro* cell free experiment Cdc42 can act as a competitive inhibitor of ROS production by Rac-1 and Rac-2 activation of cytochrome b_{558} . The cyt- b_{558} -binding domain of Cdc42 resulted in competitive binding with Rac2 (Diebold et al., 2004).

The formation of filopodia enables cells to sense their environment in a particular direction. Cells can then form lamelopodia fronts, with a gradual shift in the dominance between these fronts towards a single frontal region in the direction of movement. Cdc42 has been shown to be important in the formation of lamelopodia by restricting where lamelopodia form (Srinivasan et al., 2003). The ability to shift fronts is important for cells to enable them to turn and migrate in the desired direction. The persistence measure reflects the ability of the cells to turn and achieve directionality. Thus it was in keeping with their lack of chemotaxis that Nox2^{-/-} BMM demonstrated more convoluted migratory paths.

Thus the results established an important role for the ROS from Nox2 in the establishing directionality in BMM migration towards CSF-1.

3.4.3 *MIGRATION- SPEED AND PERSISTENCE*

MAIN FINDING:

- The innate persistence in the direction of random motion in BMM is increased in the absence of Nox2.

- Speed and persistence of directed motion is reduced in the absence of Nox2.
- Downstream Nox2 signalling is mediated by ERK1/2.

The experiments assessing the random motion of the BMM showed a significant reduction in the numbers of Nox2^{-/-} BMM reaching the set horizon and an increased intrinsic persistence in direction of motion. Random motion allows cells to explore their environment. The increased intrinsic persistence observed in Nox2^{-/-} BMM under random migration suggested that the loss of Nox2 could result in a reduction in the cells ability to turn and explore their environment. Indeed Pankov et al (Pankov et al., 2005) had demonstrated that total Rac1 activity was important in determining whether random cell migration followed a more intrinsic random or directionally persistent pattern of motion. The data here suggests that, at least in part, some of these regulatory functions of Rac1 could be through Nox2. Moreover the loss of Nox2 had also significantly reduced the distance the BMM randomly migrate to explore their immediate environment, following CSF-1 stimulation. Taken together the data demonstrated an important role of for Nox2 in BMM migration.

In contrast to random motion, directional migration moves cells rapidly between points. An important result was the significant reduction in the cellular speed of migration in Nox2^{-/-} BMM under directed motion. The speed reflects an integrated measure of the rate of actin turnover in the cytoskeleton, actin-myosin contractility and integrin adhesion, detachment and their recycling by endocytosis and vesicular transport.

The molecular mechanism for the Nox2 dependency in the speed of BMM migration is not established. Indeed many of the proteins involved in the control of actin cytoskeleton reorganisation are redox sensitive such as PTENS and PI3K (Kim et al., 2008). (Figure 3.2) Lamellipodia in moving cells require cycles of actin polymerization and depolymerisation. Rac stimulates actin polymerization by several mechanisms, including NADPH oxidase mediated ROS production (Moldovan et al., 2000). The relation between the actin cytoskeleton and ROS seems to work both ways. Cortactin, an actin-binding protein that has traditionally been found to regulate polymerization of the actin cortex, has also been shown to mediate p47^{phox} translocation to the membrane during angiotensinII induced activation of NADPH oxidase (Touyz et al., 2005). Moreover, actin activates Nox2 in neutrophils in a cell-free system, implying a direct effect on NADPH oxidase enzyme activity, and destabilization of the actin cytoskeleton robustly enhances the neutrophil respiratory burst activity (Bengtsson et al., 2006; Morimatsu et al., 1997). A more complete understanding of this bidirectional relation between NADPH oxidases and the actin cytoskeleton may shed further light on how it mediates migration.

The significantly reduced phosphorylation of ERK1/2 was in line with its important role in cellular migration and that of Nox2 in the activation of Ras/Raf/MEK/ERK signalling cascade downstream from the tyrosine receptors. ERK1/2 localise to the cell membrane (Glading et al., 2001) and to focal adhesions (Fincham et al., 2000) and promote lamellipodium formation and spreading in epithelial cells (Ishibe et al., 2004). Adhesion turnover is important for cellular migration (Chiarugi et al., 1998; Rigacci et al., 2002) and ERK has been shown to be important in its regulation

(Brahmbhatt and Klemke, 2003; Turner et al., 2001). Smith et al found that ERK1/2 activity was reduced in PAK1^{-/-} BMMs which displayed spreading defects compared with WT BMMs thus suggesting that optimal activation of ERK1/2 is required during BMM spreading (Smith et al., 2008). The observed reduced activation of ERK1/2 in the Nox2^{-/-} BMM following CSF-1 stimulation suggesting a possible mechanism whereby Nox2 generated ROS is able to modulate the downstream response via activation of ERK.

3.4.4 MIGRATION: ALTERNATIVE NADPH OXIDASE

Nox4 has also recently been found to be a key player in the regulation of stress fiber formation and focal adhesion turnover in VSMCs (Clempus et al., 2007). Poldip2 is a recently reported new regulator of Nox4 (Lyle et al., 2009). Poldip2 is an activator of Nox4 mediated ROS production in VSMCs which can negatively affect focal adhesion turnover and inhibit VSMC migration. These findings suggest a potentially novel mechanism of local ROS production by which focal adhesion turnover is coordinated. Certainly a role of Nox2 in the regulation of such adhesion formation in BMM could explain the difference observed in their shape and then in their speed and persistence. Further studies of difference in the expression of integrins would increase the understanding of the exact underlying mechanism whereby the loss of Nox2 results in a reduction in the speed of migration in BMM.

Schroder et al (Schroder et al., 2007) demonstrated in rat SMC that inhibition of Nox4 with siRNA did not affect migration to β FGF agonist stimulation. However the inhibition of Nox1 significantly blocked migration. The migration in these experiments was assessed by a scratch wound assay which gives a measure of cellular migration but not of the individual

components such as the speed, persistence or chemotaxis, unlike in the Dunn chamber. Nevertheless the experiment demonstrated an important role of Nox1 in the migration of VSMC to β FGF agonist stimulation. Similarly Haurani et al (Haurani et al., 2008) in a transwell set-up showed that Nox4 promotes AngII induced myofibroblast migration. Again, the transwell set-up did not enable the migration to be investigated in any more detail.

3.4.5 SUMMARY

In summary, in order to initiate cardiac fibrosis and hypertrophy the migration of macrophages into tissue is an important initial step. However the loss of Nox2 results in significant reduction in the random migration of BMM. On interrogating the BMM towards a directed target the loss of Nox2 proved crucial as its loss resulted in the complete loss of chemotaxis. Nox2 was also important in the BMM speed and persistence towards a CSF-1 gradient with significant reductions in both. This loss of Nox2 also manifested itself in a reduced ERK1/2 phosphorylation and spreading responses to CSF-1 stimulation. As macrophage infiltration is an important first step in initiating cardiac fibrosis and hypertrophy the defect in the migration of Nox2^{-/-} BMM could explain the reduction in cardiac hypertrophy and fibrosis observed in Nox2^{-/-} mice following AngII stimulation. Thus the Nox2 in the infiltrating cells, namely the BMM, could be a critical cell source for the Nox2. Thus in the next chapter the effects of AngII exposure on WT and Nox2^{-/-} BMM chemotaxis and migration was investigated.

4 EFFECT OF NOX2 ON BMM SHAPE, SIZE AND MIGRATION TO CSF-1 STIMULATION FOLLOWING ANGIOTENSIN II INCUBATION

4.1 INTRODUCTION

4.1.1 ROLE OF ANGII IN THE PATHOGENESIS OF CARDIAC FIBROSIS

Pathological AngII signalling in vascular, endothelial and cardiac cells promotes inflammation and altered vasoreactivity, migration and fibrosis. These effects ultimately contribute, at least in part, in causing hypertension, atherosclerosis, and heart failure. Improved clinical outcomes after treatment with ACE inhibitors and ARBs confirm the importance of AngII in the pathogenesis of these disease (Garg and Yusuf, 1995; Igarashi et al., 2001; Yusuf et al., 2000).

The development of cardiac fibrosis, as part of hypertensive heart disease, results from the cellular deposition of extracellular matrix and the infiltration of macrophages, as described in the introduction [section 1.3.3]. A number of studies and experimental models of atherosclerosis and hypertension have shown that RAS blockade ameliorates monocytes/macrophage infiltration (Capers et al., 1997; Dol et al., 2001). In keeping with the role of AngII in cardiac fibrosis, AngII has also been implicated in ECM formation and regulation (Matsubara et al., 2000; Touyz et al., 2001b), in regulation of collagen (Kato et al., 1991; Mifune et al., 2000) and proteoglycan synthesis (Evanko et al., 1998; Iozzo, 1998; Sasamura et al., 2001). Furthermore the production of matrix metalloproteinases and breakdown of collagen IV is also modulated by AngII (Libby and Lee, 2000).

These effects of AngII are humoral and occur independently of the physical blood pressure effects. Hence cardiac fibrosis has been found to develop in both the left and right ventricles, on biopsies of myocardial tissue, despite the right heart pumping blood against a reduced afterload and not developing cardiac hypertrophy (Amanuma et al., 1994). Studies have shown that the blockade of AngII or its action results in reduced fibrosis. As such Tokuda et al (Tokuda et al., 2004) demonstrated that a sub-depressor dose of candesartan (an angiotensin II receptor blocker), in mice who had supra-renal aortic banding, markedly ameliorated perivascular fibrosis. Also, AngII infusion increased aortic atherosclerosis and aneurysm formation again independent of blood pressure. Hence the pathophysiological importance of AngII in hypertensive heart disease cannot be overstated.

4.1.2 ROLE OF ANGII IN CELLULAR MIGRATION

The infiltration of macrophages is an important early event in the development of AngII induced cardiac fibrosis. This was demonstrated by the blockade of MCP-1 or ICAM-1, by way of neutralising antibodies (Kuwahara et al., 2003; Kuwahara et al., 2004). This inhibition of macrophage recruitment and tissue infiltration prevented the onset of cardiac fibrosis.

AngII has been shown in a number of studies to induce cellular migration via MAPK activation (Kyaw et al., 2004; Ohtsu et al., 2005; Xi et al., 1999). In keeping with this Yoshizumi et al (Kyotani et al., 2010) demonstrated that olmesartan inhibited AngII induced VSMC migration. However, the downstream signalling and subsequent cellular migration induced by AngII has been shown to be more complex. Thus aldosterone has been shown to act synergistically with AngII to stimulate VSMC migration.

This cross talk between aldosterone and AngII is complex, involving downstream c-Src regulated NADPH oxidase activation, resulting in Rho/Rho kinase activation controlling cellular migration (Montezano et al., 2008). This synergistic effect on cellular migration could explain, at least in part, the several lines of evidence suggesting a complex interaction between AngII and aldosterone in-vivo from our laboratory and others (Iglarz et al., 2004; Johar et al., 2006). Along with inducing migration AngII has also been demonstrated to augment cellular migration towards other chemoattractants. Thus VSMC pre-treated for 48 or 72 hours with AngII demonstrated significantly more migration towards PDGF. This effect was ERK1/2 dependent and was inhibited by the AT1 receptor blockade (Blaschke et al., 2002). Thus AngII has an important role in inducing cellular migration which is an essential cellular event in the development of cardiac fibrosis.

4.1.3 ROLE OF NOX2 IN THE PATHOGENESIS OF ANGII INDUCED CARDIAC FIBROSIS

Many studies, in particular animal studies of AngII induced hypertensive heart disease causing cardiac hypertrophy and fibrosis, have shown an important role for Nox2 (Bendall et al., 2002a; Byrne et al., 2003; Johar et al., 2006), as summarised in the introduction [section 1.9]. Thus in mouse models of hypertensive heart disease simulated with AngII infusion, our laboratory has shown that Nox2^{-/-} mice had markedly reduced fibrosis (Bendall et al., 2002a; Byrne et al., 2003; Johar et al., 2006). Furthermore Johar et al (Johar et al., 2006) showed that the profibrotic effects of AngII were inhibited by the antioxidant N-acetyl-cysteine, consistent with the role

of ROS in this process. Nox2 has also been implicated in aldosterone induced myocardial fibrosis (Johar et al., 2006). Also previous studies using global Nox2 knockout mice have shown Nox2 to be important in the development of cardiac hypertrophy in response to a low sub-pressor dose of AngII (Bendall et al., 2002a) and also at pressor doses (Byrne et al., 2003). Thus these studies established an important association between AngII stimulation, NADPH oxidase and the development of cardiac fibrosis and hypertrophy. However these studies were not able to ascertain the cell source where the effect of the Nox2 was crucial for the development AngII induced hypertensive heart disease. In chapter 3 Nox2 was shown to be crucial in BMM migration towards CSF-1 thus establishing BMM as a possible important cell source for Nox2 in the development of AngII induced cardiac fibrosis and hypertrophy.

4.1.4 DOWNSTREAM ANGIOII SIGNALLING AND NOX ACTIVATION

Once AngII binds to its receptor it activates a series of signaling cascades. Most of the known physiological effects of AngII are mediated by its receptor angiotensin type 1 receptor (AT1R) (Matsusaka and Ichikawa, 1997). This receptor is widely distributed in all organs, including liver, adrenals, brain, lung, kidney, heart and vasculature. Once AngII binds to the AT1R, it activates a series of signaling cascades, which in turn regulates the various physiological effects of AngII.

One well-established mechanism by which AngII signaling occurs involves the classic G protein-mediated pathways (Ushio-Fukai et al., 1998). These proteins activate further downstream effectors such as phospholipase C (PLC), phospholipase A₂ (PLA₂) and phospholipase D (PLD) (Ushio-Fukai

et al., 1999). Activation of PLC produces inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to its receptor on sarcoplasmic reticulum opening channels to allow efflux of calcium into the cytoplasm causing eventual cellular contraction. DAG activates PKC which participates in the activation of Ras/Raf/MEK/ERK pathway. PLD activation results in hydrolysis of phosphatidylcholine (PC) to choline and phosphatidic acid (PA). PA is rapidly converted to DAG leading to sustained PKC activation. PLA_2 activation leads to the production of arachidonic acid (AA) and its metabolites. These derivatives of AA not only contribute to cellular contraction but AA itself can also act by activating NADPH oxidase (Block et al., 2006). Thus G protein mediated pathways contribute to cellular contraction and activate downstream proteins that enhance migration related signalling.

In addition to signaling through heterotrimeric G proteins, AT_1 receptors have also been shown to activate monomeric small G proteins. The small G proteins were introduced in chapter 3 comprising of RhoA, Rac1 and Cdc42 amongst others, that act as molecular switches to regulate cellular responses (Laufs and Liao, 2000). AngII stimulated Rac1 participates in cytoskeletal organization, cell growth, inflammation and regulation of NADPH oxidase. In VSMC AngII stimulated Rac1 acts upstream of p21-activated kinase and JNK.

An alternate mechanism by which AngII signals further downstream is by activating MAPKs, including ERK1/2, JNK and p38MAPK. AngII can differentially activate the three major members of the MAPK family (Touyz et al., 1999; Touyz et al., 2001a) and the events further downstream are numerous and heterogeneous. AngII stimulates phosphorylation of many

non-receptor tyrosine kinases including c-Src family kinases , Ca²⁺-dependent tyrosine kinases (e.g Pyk2) , focal adhesion kinase (FAK) and Janus kinases (JAK). In addition, AngII can cross-talk with several receptor tyrosine kinases, via AT₁R activation, including EGFR (Ushio-Fukai et al., 1998), PDGF (Heeneman et al., 2000) and insulin receptor. The induction of the above mentioned pathways is tightly regulated. In patients with overstimulated RAAS or enhanced responsiveness to AngII these pathways may initiate and propagate pathological events. The role of tyrosine kinases in AngII-mediated signaling has been extensively reviewed elsewhere (Berk and Corson, 1997; Kim and Iwao, 2000; Matsusaka and Ichikawa, 1997).

Interestingly many of the effects of AngII are mediated by ROS (Griendling et al., 2000). These have been demonstrated by the use of antioxidants, where the use of SOD or tempol corrected the AngII induced elevation in blood pressure with a decrease in superoxide production (Laursen et al., 1997; Nishiyama et al., 2001). ROS are also known to directly affect important regulators of cell migration such as PTEN, FAK or Src (Chiarugi et al., 2003; Kwon et al., 2004; Li et al., 2008). A growing body of data point to the key role of ROS production by NADPH oxidase in the control of cell migration and cytoskeletal reorganization (Moldovan et al., 2000; Ushio-Fukai, 2006). NADPH oxidase has been shown to be an important source of ROS formation following AngII stimulation in cultured VSMC (Griendling et al., 1994). Infusion of AngII has been shown to induce hypertension associated with an increase in the NADPH oxidase derived superoxide production (Rajagopalan et al., 1996a). This increased activity of NADPH oxidase was associated with an increase in the expression of p22^{phox}, levels of which decreased following the use of SOD.

The signalling pathways in VSMC have been the most extensively studied, where it has been shown that AngII regulates NADPH oxidase activity in a biphasic manner. The initial phase occurs rapidly, within 30-seconds, and involves the activation of PKC and the mobilization of calcium to initiate p47^{phox} phosphorylation. The second plateau phase which occurs over approximately 6-hours acts through c-Src and PI3-K to activate Rac1 and other NADPH oxidase subunits (Seshiah et al., 2002). AngII can also regulate NADPH oxidase by increasing the expressions of its subunits and influencing the rate of their association. As such in human VSMCs increased levels of p47^{phox} mRNA has been shown along with increased levels of phosphorylation and translocation of p47^{phox} to the membrane following AngII stimulation (Touyz et al., 2003; Touyz et al., 2004). In isolated rat VSMCs, interfering with p22^{phox} antisense transfection resulted in a reduction in AngII induced superoxide production (Ushio-Fukai et al., 1996). The overexpression of p22^{phox} targeted specifically to VSMC potentiated AngII-induced vascular hypertrophy the effect of which was prevented by the treatment with ebselen an antioxidant, thus verifying ROS as a direct activator (Weber et al., 2005). In other cell types AngII has been found to regulate NADPH oxidase activity by again regulating the expression of its subunits. Thus in CMEC, p22^{phox} expression and subsequent ROS production (Xie et al., 2001), the expression of Nox2 and Nox4 in human umbilical vein and the expression of Nox1, 2 and 4 and p22^{phox} in endothelial cell from rat aorta have been shown to be increased following treatment with Ang II (Higashi et al., 2003; Yamagishi et al., 2005).

In summary, following AngII stimulation the downstream signalling pathways are extensive and complex. This enables the downstream signalling

of AngII to be modulated, for example by NADPH oxidase, and also allows for AngII to modulate the downstream signaling of other agonists such as CSF-1.

4.1.5 SUMMARY

Taken together the evidence suggests macrophage migration and infiltration into the heart is crucial for the development of hypertensive heart disease secondary to AngII stimulation. Evidence from our laboratory amongst others has shown Nox2 to play an important role in AngII induced cardiac fibrosis. In chapter 3 Nox2 was shown to be crucial in the migration of BMM towards a CSF-1 gradient thus establishing the Nox2 in BMM as an important cell source. However the exact effect that the presence of AngII may have on this BMM migration, especially in the absence of Nox2, is not established. The current chapter aims to investigate the differences the presence of AngII may have on the cellular migration of BMM, especially in the absence of Nox2.

4.2 METHODS

The methods in this chapter are similar to those in chapter 3. Consequently the methods are only described here in summary with details given of the differences in the methodology where they arise.

4.2.1 ISOLATION AND CULTURE OF MOUSE PRIMARY BMMs

The murine femoral bone were harvested and the marrow flushed using a 21-gauge needle with 5 mL of macrophage starve medium (section 8.2.2). After centrifugation the pellet was resuspend in 5 mL of macrophage starve medium, counted and seeded at 2×10^5 cells/cm² density onto a 10cm tissue culture petri dishes for 3 days in macrophage growth media.

After 3 days the non-adherent population of cells containing the monocytes were removed. The cells were centrifuged, resuspended and counted and then cryogenically frozen in growth medium. When required the cells were defrosted and seeded onto 6-cm bacterial culture plates in 5 mL of growth medium at a density of 10 cells/mL. The cells were then incubated for a further 5 days without refeeding. AngII was added to the appropriate cells at a concentration of 10^{-7} M as previously used in our laboratory. This was replenished after 2 days to maintain the concentration of AngII. The cells in general remained in suspension for approximately 4 days. The differentiated BMMs then became adherent and were harvested on day 5 for experimentation. The cells were harvested off the dish with the dissociation solution Versene.

4.2.2 DUNN CHEMOTAXIS CHAMBER EXPERIMENTS

4.2.2.1 Preparation

The Dunn Chemotaxis Chambers (DCC) were washed and prepared from storage as before (section 3.2.3.2). Care was taken to invert the coverslip with the AngII incubated cells attached onto the chamber, such that the inner well was completely covered with a gap left in the outer well. The inner well contained macrophage starve media with AngII. (Figure 4.1 B) Once the coverslip was in place, the chamber was sealed on three sides with wax and the outer well was drained from the fourth side (gap) with Whatman paper. 100 μ L of macrophage growth media with AngII was then added into the outer well through the gap. Thus there was a CSF-1 gradient and no AngII gradient across the bridge in the Dunn chamber. (Figure 4.1 B & C) The final edge was quickly waxed to ensure that the gap was completely sealed. The DCC was immediately placed onto the microscope stage and filming started. (also see section 3.2.3.2)

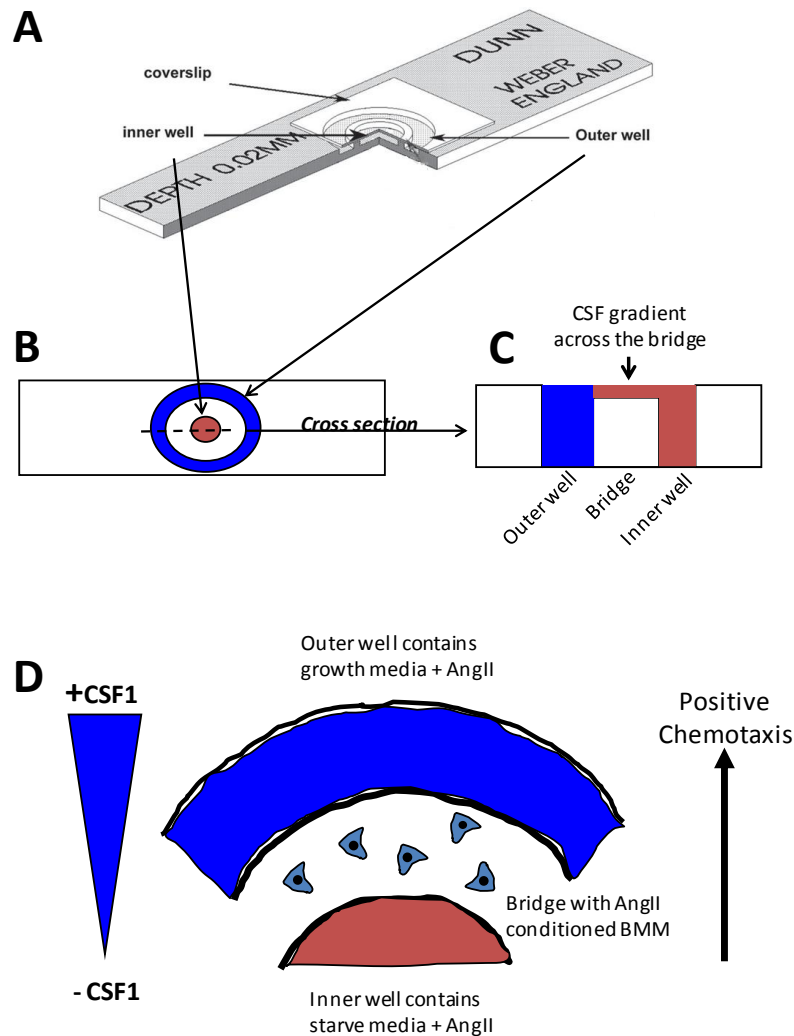


Figure 4-1 Preparation of the Dunn Chamber

A: Cover slip with AngII conditioned BMM was placed onto the Dunn chamber. B: The outer chamber contained growth media (+CSF) with AngII while the inner chamber was filled with starve media (no CSF) with AngII. C: Thus there was no AngII gradient across the bridge. The cells are exposed only to the CSF-1 gradient. D: Positive chemotaxis of the AngII conditioned BMM towards the CSF-1 gradient was recorded and analysed.

4.2.2.2 Time-Lapse Microscopy and Migration Analysis

The assembled chamber was placed on the heated stage and a region of the bridge was selected for recording and the image orientated such that the source of chemoattractant, namely the CSF-1, in the outer well was at the top of the screen. (Figure 4.1 D) The cells were filmed for 18hrs and subsequently tracked and analysed using Mathematica. (also see section 3.2.3.3)

4.2.3 STAINING

The AngII incubated BMM were seeded onto the coverslips at 2×10^5 cells per coverslip. The cells were allowed to settle and then those for starvation and/or restimulation were starved of CSF-1 by overnight replacement of AngII-containing growth medium with AngII containing starve media. Following CSF-1 starvation the cells for restimulation were stimulated with CSF-1 by the reintroduction of growth media containing AngII for 5mins. Thus the response of the cells to CSF-1 was assessed in the presence of AngII. The cells were then washed with PBS and fixed with 1% paraformaldehyde for staining.

The cells on the coverslips were permeabilised with methanol and blocked with 5% BSA. The cells were stained for actin using phalloidin-FITC (1:1000) made in 1%BSA. The staining was visualised under fluorescent microscopy and images taken. The images were analysed using Image-J where the cells were outlined to obtain their shape and size. Five random fields were chosen per slide. (also see section 3.2.4)

4.2.4 WESTERN BLOTTING

The cells were seeded onto 6 well plates in macrophage growth medium containing AngII. The growing cells did not have their media changed. Those for starvation had their media changed to macrophage starve media containing AngII. After overnight starvation of CSF-1, macrophage growth medium containing AngII was added to cells for restimulation for 5, 10 15, 30 or 60mins following which the wells were washed and lysis solution added. The 6 well plates were kept on ice, the wells scraped, samples removed and placed into eppendorf tubes. The samples were spun at 12000rpm for 30 mins and the supernatant removed to a new eppendorf. To denature the protein, samples were heated to 100°C for 3mins, sample buffer was added and the sample frozen and stored. Protein expression was then analysed with western blotting as described earlier.(also see section 3.2.5)

4.3 RESULTS

4.3.1 *Nox2^{-/-} BMM IN THE PRESENCE OF ANGIOTENSIN II HAVE A SIGNIFICANTLY INCREASED SPREAD AREA.*

The average spread area of growing WT BMM in the presence of AngII was smaller.(Figure 4.2) In complete contrast, in the presence of AngII, Nox2^{-/-} BMM showed a significant increase in their growing spread area by 22% (0.057AU; p=0.04). As a result growing BMM spread area, in the presence of AngII, was significantly (p<0.001) greater in Nox2^{-/-} BMM compared with WT BMM.

In the presence of AngII cell elongation decreased by a similar amount in both WT and Nox2^{-/-} BMM. Thus growing cellular shape, in the presence of AngII, was not affected by the absence of Nox2.

AngII incubation of growing WT and Nox2^{-/-} BMM resulted in an altered phenotype. The presence of AngII had resulted in both WT and Nox2^{-/-} BMM becoming more rounded in shape. However, while growing WT BMM showed a decrease in their cellular spread area, Nox2^{-/-} BMM showed a significant increase. Thus, the data suggest that BMM can differentially regulate their cellular shape and size. Also that AngII signalling via Nox2 reduces cellular size which in the absence of Nox2 results in stimulation of other signalling pathways which has the opposite effect.

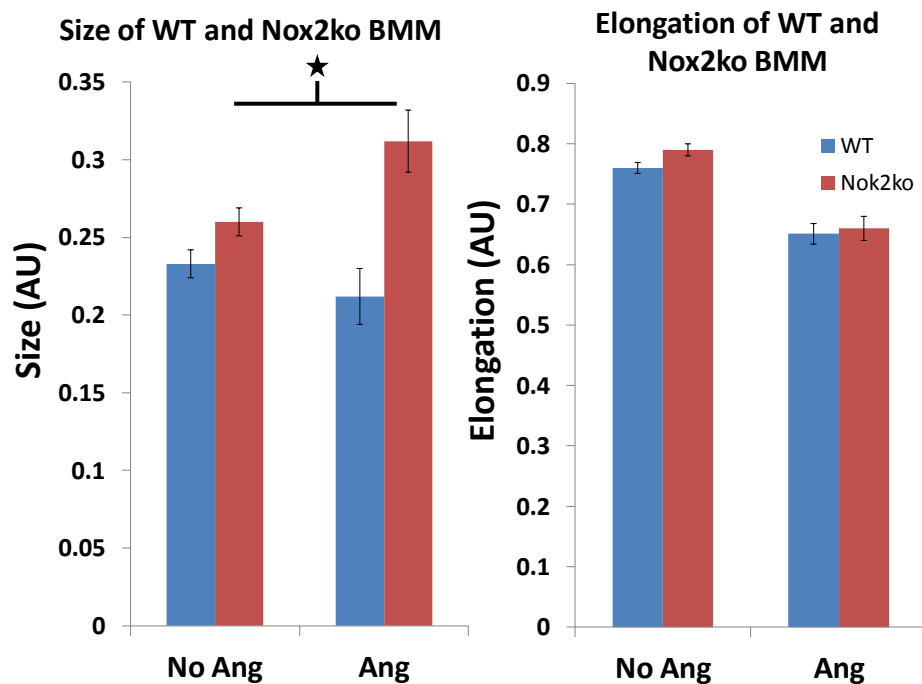


Figure 4-2 Difference in the spread area and elongation between growing WT and Nox2^{-/-} BMM following AngII incubation.

Growing BMM showed an important role for Nox2 in the regulation of their spread area following AngII incubation. WT BMM decreased their spread area in contrast to Nox2^{-/-} BMM which showed a significant increase in their spread area in the presence of AngII. This opposing response in cellular size is in contrast to the regulation of cellular shape in which the more elongated phenotype of Nox2^{-/-} BMM was rescued such that both groups of cells had a similar, more rounded, shape in the presence of AngII. (★= $p < 0.05$, N=150 WT or Nox2^{-/-} cells)

4.3.2 Nox2^{-/-} BMM SHOW LESS CSF-1 INDUCED CELLULAR SPREADING IN THE PRESENCE OF ANGII

In chapter 3 CSF-1 starvation had resulted in a 37% reduction in WT BMM spread area and a 38% reduction in Nox2^{-/-} BMM spread area (p=ns). It should be noted that the Nox2^{-/-} BMM had a larger absolute growing spread area than WT BMM. The spread area, in the presence of AngII, showed a similar reduction in both WT and Nox2^{-/-} BMM of 23% and 36% respectively. Thus the ability of WT and Nox2^{-/-} BMM to relatively change their spread area, following CSF-1 starvation, was unaffected by the presence of AngII. However it is noteworthy that the AngII incubated Nox2^{-/-} BMM had a significantly (p=0.048) larger absolute starved spread area from that observed in WT, Nox2^{-/-}, and AngII incubated WT BMM. (Figure 4.3). Taken together the data demonstrated that, though WT and Nox2^{-/-} BMM showed a relatively similar response to CSF-1 starvation, in the presence of AngII, the initial larger growing spread area observed in AngII incubated Nox2^{-/-} BMM (Figure 4.2), was not completely reversed following CSF-1 starvation, as was observed in Nox2^{-/-} BMM without AngII. (Figure 3.7)

The re-introduction of CSF-1 to AngII incubated WT BMM showed a significant increase in their spread area. The area increased by 54% (0.088 AU; p<0.001) which was less than the 79% response seen in WT BMM in the absence of AngII. However this reduced response, by 25%, was not statistically significant (p=0.09).

In contrast, the re-introduction of CSF-1 to AngII incubated Nox2^{-/-} BMM showed only a borderline significant increase in their spread area. The area increased by only 14% (0.03 AU; p=0.05) as compared to the 55% response seen in Nox2^{-/-} BMM in the absence of AngII. This reduced

response, by 41%, was statistically significant ($p < 0.001$). Thus in Nox2^{-/-} BMM the presence of AngII had further reduced the spreading effect by CSF-1.

Thus in summary the data showed that, in the presence of AngII, Nox2 is important in CSF-1 induced cellular spreading. In the presence of AngII, WT BMM had a reduced cellular spreading response to CSF-1 stimulation. However the loss of Nox2 not only resulted in a reduced ability to contract fully on CSF-1 starvation but also prevented a significant cellular spreading response.

Difference in increase in spread area following 5mins CSF-1 stimulation in WT and Nox2ko BMM with and without ANG incubation

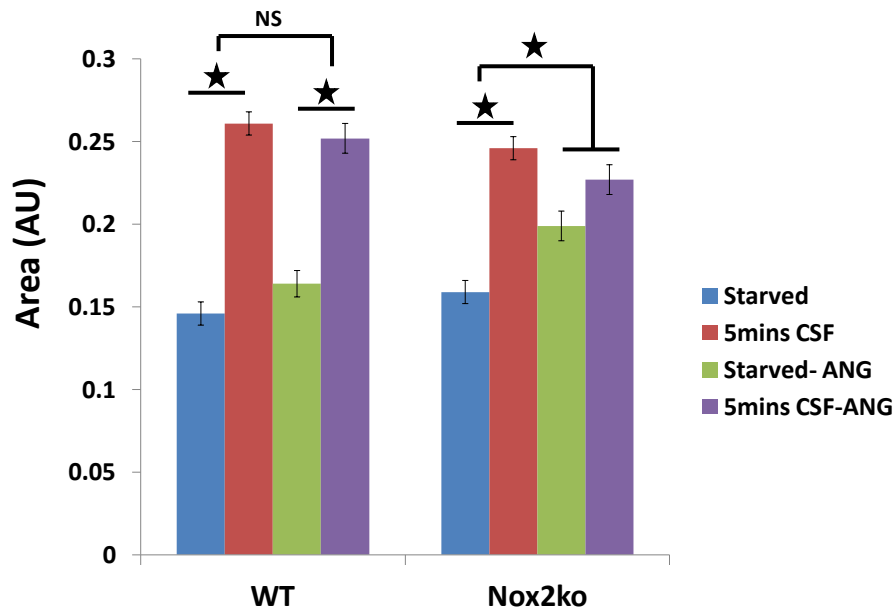


Figure 4-3 Difference in the increase in spread area following 5mins CSF-1 stimulation in WT and Nox2ko BMM with and without ANG incubation.

Incubation of WT BMM with AngII resulted in a non-significant reduction in the cellular spreading in response to CSF-1 stimulation. (WT:79% vs WT AngII:54%) In contrast the response in the Nox2^{-/-} BMM, following AngII incubation, of just 14% was only borderline significant (p=0.05). The cellular spreading response, following incubation with AngII, was a significantly reduced (Nox2^{-/-}: 55% vs Nox2^{-/-} AngII: 14%). (★= p<0.05) Thus Nox2^{-/-} BMM appear to lose their CSF-1 induced cellular spreading response following incubation with AngII.

4.3.3 CSF-1 STIMULATION IN Nox2^{-/-} BMM INDUCE A LARGER REDUCTION IN CELLULAR ELONGATION ONLY IN THE PRESENCE OF AngII

The presence of AngII did not affect the ability of WT and Nox2^{-/-} BMM to elongate following CSF-1 starvation, similar to their response in the absence of AngII. (see section 3.3.3)

The presence of AngII did not significantly affect the ability of WT BMM to change shape following restimulation with CSF-1. (Figure 4.4) However, in the presence of AngII, the Nox2^{-/-} BMM shortened significantly more [0.06AU (p=0.01)] than WT BMM. Thus the data suggests that AngII does not affect the cellular shape changes induced by CSF-1 stimulation and that the response elicited by AngII in Nox2^{-/-} BMM is non-Nox2 dependent signalling pathways and is peculiar to the Nox2^{-/-} BMM.

Difference in the decrease in elongation following 5mins CSF-1 stimulation in WT and Nox2ko BMM with and without ANG incubation

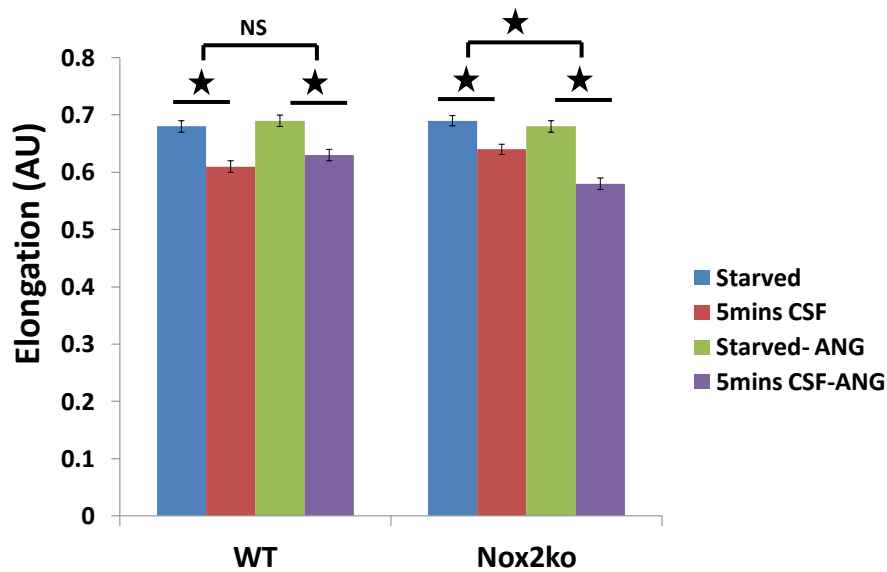


Figure 4-4 Increased reduction in the elongation of Nox2^{-/-} BMM incubated with AngII following 5mins CSF-1 stimulation

The decrease in elongation in WT BMM following CSF-1 stimulation is unchanged in WT BMM incubated with AngII or with the loss of Nox2. However the absence of Nox2 in BMM incubated with AngII resulted in a larger decrease in their elongation following CSF-1 stimulation. This is suggestive of a more complicated downstream interplay between Nox2 and AngII signalling.

In summary the smaller and more rounded phenotype observed in WT BMM in the presence of AngII is significantly changed in the absence of Nox2 resulting in a larger growing spread area. Reduction in BMM's ability to reduce and increase cellular spread area following CSF-1 starvation and stimulation in the presence of AngII is significantly enhanced in the absence of Nox2. The presence of AngII or the absence of Nox2 did not significantly influence cellular shape changes following CSF-1 stimulation. However in combination the presence of AngII and the absence of Nox2 demonstrated a

hitherto unobserved cellular shape change following CSF-1 stimulation due to AngII signalling via non-Nox2 downstream signalling pathways.

4.3.4 Nox2^{-/-} BMM MAINTAINED A LACK OF CHEMOTAXIS TOWARDS A CSF-1 GRADIENT IN THE PRESENCE OF ANGII

In chapter 3 Nox2^{-/-} BMM showed a loss of chemotaxis towards a CSF-1 gradient. The presence of AngII did not change this response in WT and Nox2^{-/-} BMM. Thus WT BMM, in the presence of AngII, continued to demonstrate significant positive chemotaxis towards a CSF-1 gradient whereas the Nox2^{-/-} BMM showed a similar lack of chemotaxis. (Figure 4.5)

The data would therefore suggest that any stimulation of the downstream signalling in the presence of AngII is redundant with regards to influencing the positive chemotaxis as observed in WT BMM. More importantly any such changes were not able to rescue the lack of chemotaxis observed in the absence of Nox2.

4.3.5 ABSENCE OF NOX2 REVERSED THE EFFECT OF ANGII ON SPEED OF MIGRATION

In the presence of AngII WT BMM showed a significant ($p < 0.001$) decrease in their speed of migration towards a CSF-1 gradient. (Figure 4.5) The speed was reduced to the level observed in Nox2^{-/-} BMM without AngII stimulation ($p = 0.2$). In contrast, with the absence of Nox2 a significant ($p < 0.001$) increase was observed in the speed of migration. (Figure 4.5) The speed increased to a value similar to that observed in WT BMM without AngII stimulation ($p = 0.1$).

These data suggests that following AngII stimulation, the downstream Nox2 dependent signaling resulted in a reduction in cellular speed of migration, towards a CSF-1 gradient. In the absence of Nox2 AngII stimulation increased the speed of migration towards a CSF-1 gradient. Hence this implicated the signalling pathways stimulated, independent of Nox2, by AngII as being pro kinetic towards CSF-1.

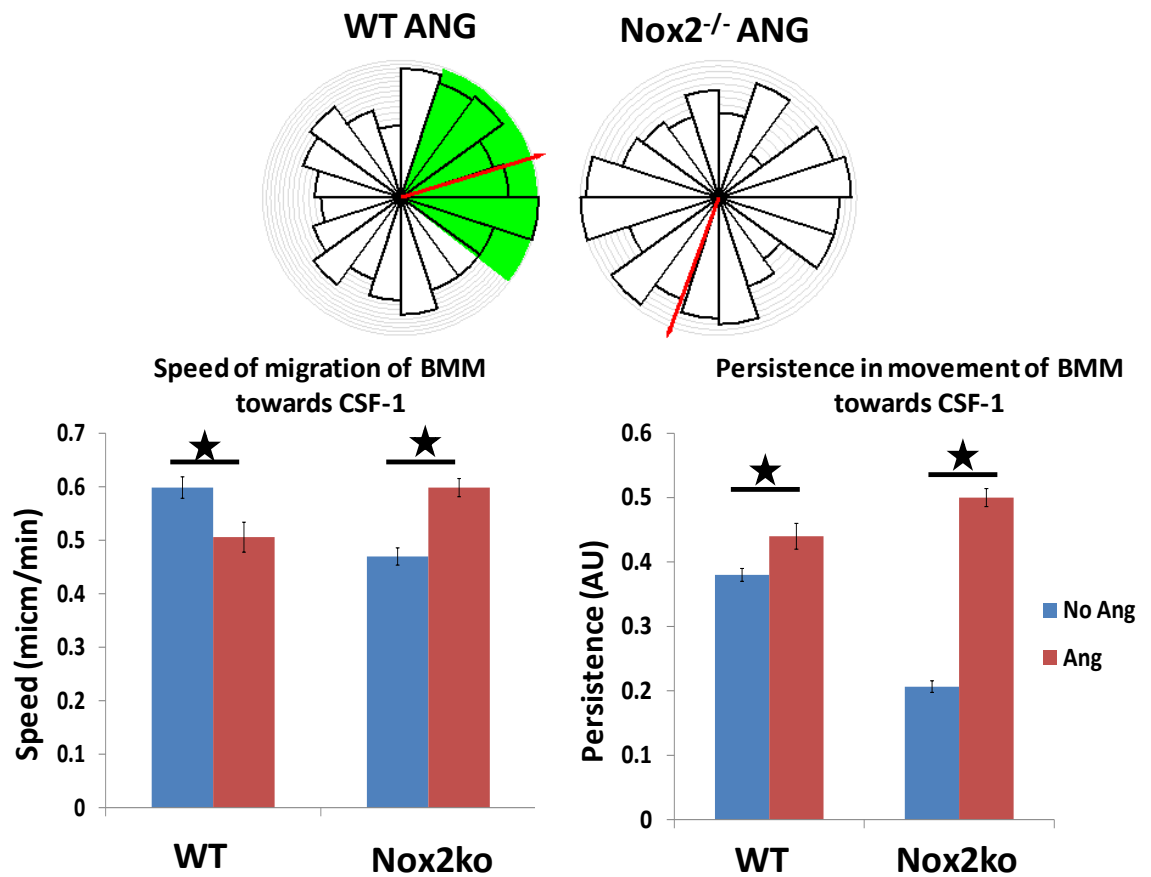


Figure 4-5 The chemotaxis speed and persistence in the motion of WT and Nox2^{-/-} BMM

WT BMM incubated with AngII demonstrated positive chemotaxis which was not observed in the Nox2^{-/-} BMM. In the presence of AngII the speed of migration decreased and the persistence increased in WT BMM. In the absence of Nox2, AngII stimulation resulted in an increase in the speed of migration and the persistence showed an even larger increase. Thus in the presence of AngII, Nox2 dependent signalling, reduces the speed and attenuates the increase in persistence towards a CSF-1 gradient. Thus both speed and persistence show increases in the absence of Nox2 following BMM incubation with AngII. (N= 20 WT or Nox2^{-/-} tracks)

4.3.6 PRESENCE OF NOX2 ATTENUATES ANGII INDUCED INCREASE IN PERSISTENCE OF THE MIGRATION IN BMM TOWARDS A CSF-1 GRADIENT.

The presence of AngII significantly ($p=0.006$) increased the persistence in the migration of WT BMM. (Figure 9.5) In chapter 3 the loss of Nox2 had resulted in a significantly ($p<0.001$) decreased persistence. However in the presence of AngII, Nox2^{-/-} BMM showed a significant ($p<0.001$) and substantial increase in persistence to a value which was significantly greater than that observed in AngII incubated WT BMM ($p<0.001$). (Figure 4.5) The data imply that downstream signaling of AngII via Nox2 could possibly be inhibiting the increase in persistence, thus the absence of Nox2 resulted in a larger increase in cellular persistence. Alternatively, other non-Nox2-dependent signalling pathways may be stimulated underlying the increase.

Taken together the data suggests that the effect of AngII appears to be one where the cells migrate more slowly with more directionality towards the CSF-1 gradient. The response in Nox2^{-/-} BMM was considerably different. Given their lack of chemotaxis towards the CSF-1 gradient, the increased speed and persistence manifested itself as rapid forward and backward movements on the Dunn chamber.

4.3.7 IN THE PRESENCE OF ANG II CSF-1 STIMULATION RESULTED IN MORE ERK1 PHOSPHORYLATION IN NOX2^{-/-} BMM

WT and Nox2^{-/-} BMM in the presence of AngII showed a similar ERK1/2 and Akt phosphorylation profile to that observed in the absence of AngII. Namely the increase in phosphorylation peaked in intensity by 15mins after which it declined to baseline.

As such with AngII incubation both WT and Nox2^{-/-} BMM showed an increased level of ERK1 and ERK2 phosphorylation by 15 mins. ERK1 phosphorylation in WT BMM at 15mins was higher though this did not reach statistical significance (p=0.25). (Figure 4.6) However in Nox2^{-/-} BMM ERK1 phosphorylation increased significantly (p=0.023).

The phosphorylation of ERK2 following AngII incubation in both WT and Nox2^{-/-} BMM showed a similar significant (WT: p=0.02 and Nox2^{-/-}: p=0.05) increase by 15 mins. (Figure 4.6)

Following AngII incubation the levels of Akt phosphorylation did not show any significant difference from the levels of phosphorylation observed in WT or Nox2^{-/-} BMM in the absence of AngII. (Figure 4.6)

Thus, following CSF-1 stimulation, AngII incubation had resulted in a significantly larger increase in ERK2 phosphorylation, independent of Nox2. However with the loss of Nox2 a larger increase in the level of ERK1 phosphorylation was seen in the presence of AngII. Therefore this differential response could, in part, explain the exaggerated responses observed in Nox2^{-/-} BMM, in the presence of AngII, following CSF-1 stimulation in regards to shape changes, speed and persistence of migration.

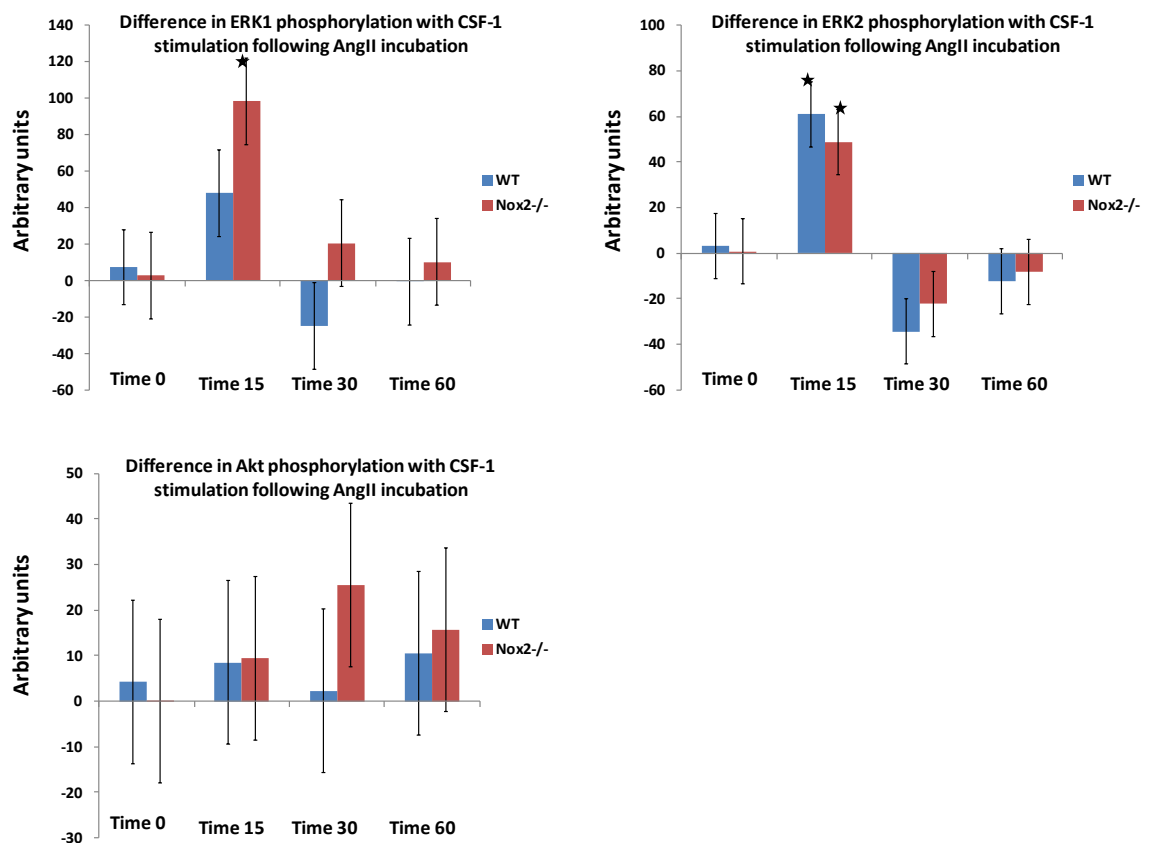


Figure 4-6 Change in phosphorylation profile following AngII incubation to CSF-1 stimulation

A significant increase in the levels of ERK1 phosphorylation at time point 15mins was seen in Nox2^{-/-} BMM following AngII incubation. The increase in ERK1 phosphorylation in WT BMM did not reach statistical significance. Significant increases in the levels of ERK2 phosphorylation was seen in both WT and Nox2^{-/-} BMM. No significant change was seen in the Akt phosphorylation. Thus following in the presence of AngII, ERK1 phosphorylation following CSF-1 stimulation could be Nox2 dependent. (★:p<0.05, N=3)

4.4 DISCUSSION

In hypertensive heart disease, the RAAS has been shown to be upregulated (Egido, 1996) and this increased exposure of cells and tissue to AngII is thought to play an important pathophysiological role. Thus AngII has been demonstrated to affect cell adhesion (Kim et al., 1996; Pastore et al., 1999; Prasad et al., 2001) and chemotaxis (Blaschke et al., 2002; Fukuhara et al., 2000; Liu et al., 2003; Montezano et al., 2008), responses important in the development of cardiac fibrosis and hypertrophy. In chapter 3 Nox2 was demonstrated to be crucial in BMM chemotaxis towards a CSF-1 gradient. In this chapter the effect of AngII exposure on this migratory response of BMM towards CSF-1 stimulation was investigated. Thus differences in the response between WT and Nox2^{-/-} BMM could explain, at least in part, in-vivo differences observed in the development of cardiac fibrosis and hypertrophy.

4.4.1 EFFECT OF ANGII ON BASELINE GROWING BMM SHAPE AND SIZE

MAIN FINDING:

- AngII effects mediated by Nox2 reduces the size of growing WT BMM.

One of the major functions of AngII is vasoconstriction which is mediated by G-protein dependent signaling pathways. Activation of AT₁ receptors leads to downstream effectors such as PLC which produces IP₃ and DAG. IP₃ binds to its receptor on the sarcoplasmic reticulum allowing a calcium efflux which results in cellular contraction by myosin light chain

kinase (MLCK). The contracted smaller, rounded phenotype of the growing WT BMM in the presence of AngII was understandable. Interestingly, this AngII mediated effect was dependent on Nox2 as the Nox2^{-/-} BMM failed to develop a similar rounded contracted phenotype. In contrast, the Nox2^{-/-} BMM showed a larger growing cellular size. This may simply reflect the effects of AngII in the absence of any downstream Nox2 modulation thus implying a crucial role for Nox2 in AngII induced cellular contraction regulating cellular size. Alternatively, it could be the result of AngII stimulation of other pathways in the altered cellular physiology observed in Nox2^{-/-} BMM. In chapter 2 Nox2^{-/-} fibroblasts were found to have a higher baseline ROS production probably due to the increased levels of Nox4 subunits (Byrne et al., 2003). This requires further study to elucidate whether the differential stimulation of downstream Nox subunits by AngII can result in contrasting cellular responses, namely cellular contraction or spreading.

4.4.2 EFFECT OF NOX2 ON THE INTERPLAY BETWEEN ANGII AND CSF-1 INDUCED CELLULAR SPREADING

MAIN FINDINGS:

- AngII incubation reduces CSF-1 induced spreading in BMM.
- Nox2 is important in determining the spreading response to CSF-1 stimulation, following AngII incubation.

Most in vitro studies of cellular migration use a single agent to stimulate migration. Cellular migration in vivo is probably a result of the

exposure to multiple promigratory and antimigratory molecules. Therefore receptor crosstalk is likely to occur and have a significant effect on migration. The experiments involving CSF-1 stimulation investigating cellular spreading or migration, allowed for a simple experiment to observe the interplay between AngII and CSF-1. As the cellular processes controlling cellular shape and size and that in cellular migration are similar, both were investigated in response to CSF-1 stimulation.

Stimulation of BMM by AngII reduced the spreading response induced by CSF-1. This interplay is in keeping with the effects of AngII observed elsewhere whereby it influences the signalling transduction mechanism induced by other agonists. A prime example of this is insulin signalling. In vivo studies in rats show that infusion of AngII induces insulin resistance (Ogihara et al., 2002; Patiag et al., 2000) and that patients with an imbalance in RAS homeostasis exhibit decreased insulin sensitivity (Kurtz and Pravenec, 2004; Nosadini and Tonolo, 2004). In the current experiment AngII stimulation decreased the CSF-1 induced cellular spreading response. Interestingly Nox2 was found to be important in this process. Thus the decreased response following CSF-1 stimulation, in the presence of AngII, was larger in Nox2^{-/-} BMM. The exact mechanism for this would require further study however the transactivation of receptor tyrosine kinases is a major mechanism by which AngII influences other signalling pathways and could therefore be a possibility. Both the receptor tyrosine kinase pathways and AngII activated G protein signalling pathways activate several common downstream pathways, of which some are redox sensitive. Hence nonreceptor activation of downstream receptor kinase signalling pathways by AngII can occur, with the possibility of Nox2 modulation. In recent years

c-Src, a tyrosine kinase, has surfaced as a key player and has been shown to be activated by AngII in a ROS dependent manner. Src kinase and its substrate such as FAK and Pyk2 have been shown to be regulated by AngII. Thus AngII can phosphorylate FAK to allow for cell adhesion and activation of cytoskeletal proteins including Pyk2 (Eguchi et al., 1999), paxillin (Leduc and Meloche, 1995) and talin (Sabe et al., 1997) all of which interact to regulate cell shape and size.

4.4.3 EFFECT OF NOX2 ON THE INTERPLAY BETWEEN ANGII AND CSF-1 INDUCED BMM MIGRATION- SPEED OF CELLULAR MIGRATION

MAIN FINDINGS:

- AngII incubation reduced the directed speed of motion towards a CSF-1 gradient in BMM.
- Nox2 is crucial for chemotaxis as AngII incubation could not rescue the loss in chemotaxis in Nox2^{-/-} BMM.
- Nox2 is not crucial for cellular speed towards a CSF-1 gradient as AngII incubation can rescue the reduction in speed.

BMM exposed to AngII had reduced speed of migration towards a CSF-1 gradient. This was in contrast to Blaschke et al (Blaschke et al., 2002) who had shown that pre-incubation of rat VSMC with 48 or 72 hours of AngII significantly increased PDGF directed migration. This effect was inhibited by AT₁ receptor blockade confirming it to be an AngII effect. Inhibition of

ERK1/2 also abolished the AngII effect on PDGF directed VSMC migration. In keeping with this, 48 hrs of incubation had resulted in an increase in expression and phosphorylation of ERK1/2 and proline rich tyrosine kinase 2 (Pyk2) and focal adhesion kinase (FAK) suggesting that changes in their levels could be responsible for the changes in migration. Taking both observations together the data would imply that the integrated effect of AngII on a tyrosine kinase receptor based chemoattractant could be cell-specific.

It is probable that in-vivo AngII production by local RAS at sites such as an atheromatous plaque could promote PDGF directed migration of VSMC. In contrast, a slower speed of migration in BMM may aid the migration of the macrophages across the endothelium into tissue thus explaining the observed reduction in the speed of migration in BMM towards CSF-1. Studies have shown that in normal rat and human hearts, ACE expression is confined almost exclusively to the endothelial cells of coronary arteries, arterioles and capillaries (Falkenhahn et al., 1995). Thus circulating monocytes attracted by tissue CSF-1, on exposure to endothelial AngII could slow down their motility, facilitating their diapedesis across the endothelium. Myocardial expression of ACE is upregulated in pressure overloaded rat ventricles and in viable myocardium following experimental myocardial infarction, resulting in more conversion of AngI to AngII (Challah et al., 1995; Hirsch et al., 1991; Schunkert et al., 1990; Wollert et al., 1994). Thus high AngII production at sites of inflammation, as in hypertensive heart disease, could slow and retain infiltrating macrophages to the site of interest. The increased exposure of resident cells, such as cardiac fibroblast, to these infiltrating macrophages would enable macrophages, by the paracrine production of TGF β , to transform the fibroblasts to myofibroblasts.

The Nox2^{-/-} BMM, despite an increased speed of migration in the presence of AngII, failed to reach the set horizon even after 18 hours of observations, thus failing to register a positive chemotactic response towards the CSF-1 gradient. The data would suggest that Nox2 remains crucial in establishing a chemotactic response towards the CSF-1 gradient as discussed in chapter 3 and that stimulation with AngII and further downstream signalling pathways, independent of any Nox2, is not able to rescue this. The data also suggests that the role of Nox2 in the regulation of cellular chemotaxis and speed of migration is different. Thus Nox2 is crucial for chemotaxis towards CSF-1 and though Nox2 is also important in the regulation of cellular speed, it is not essential. Stimulation by AngII can rescue the speed of migration, independent of Nox2. This has also been demonstrated in the literature by Sadok et al in HT29-D4 colonic adenocarcinoma cells. Nox1 down regulation inhibited cellular migration but did not affect total distance nor mean velocity of migration (Sadok et al., 2008) again demonstrating a differential effect of a Nox subunit in these processes.

The dynamic assembly and disassembly of integrin mediated adhesion and cytoskeletal reorganization are necessary for efficient cellular spreading and migration (Moissoglu and Schwartz, 2006). It was interesting that on acute stimulation with CSF-1 a smaller spreading response was observed in Nox2^{-/-} BMM, in the presence of AngII. However in the presence of AngII the migratory speed of Nox2^{-/-} BMM, towards the CSF-1 gradient, showed a marked increase. A possible explanation for this contrasting response could be due to the different Nox subunits. Thus Nox2 by its structure can rapidly produce more ROS than the other Nox subunits. Thus it is more suited to

elicit an acute response and could therefore be more important in the acute CSF-1 response, an effect which was increased in the presence of AngII. However, Nox4 functions constitutively and requires an upregulation in its expression to increase its effect. Haurani et al have demonstrated in rat adventitial myofibroblasts that AngII acting as a chemoattractant can increase migration in a concentration dependent manner and that this effect was Nox4 dependent (Haurani et al., 2008). The pre-incubation with AngII and stimulation of increased levels of Nox4 in Nox2^{-/-} BMM (Byrne et al., 2003) could explain the observed increase in speed of migration which was not seen in WT BMM, probably due to lower levels of Nox4. Thus exposure to endothelial AngII, Nox4 mediated increase in the migratory speed may hinder the need for macrophage adherence and transmigration. Further experiments would be required to verify this hypothesis, however, if true this effect of Nox4 in the Nox2^{-/-} mice would not be physiological and will be peculiar to the Nox2^{-/-} mice. A basal level of ROS may be required to keep proteins in their normal redox state. Thus some of the changes in the signaling cascades may not reflect the physiological state. Nevertheless the data suggests that the regulation of migratory speed is complicated, involving multiple pathways where different Nox subunits could function in contrasting roles.

4.4.4 EFFECT OF NOX2 ON THE INTERPLAY BETWEEN ANGII AND CSF-1 INDUCED BMM MIGRATION -PERSISTENCE IN CELLULAR MOTION

MAIN FINDING:

- AngII incubation can rescue the loss in persistence in directed motion towards a CSF-1 gradient.

In chapter 3 Nox2 was shown to be important in determining the persistence in the random migration of BMM and also in directed migration towards a CSF-1 gradient. AngII stimulation increased this persistence in the directed migration of WT BMM. The intrinsic propensity of cells to continue migrating in the same direction without turning is closely related to integrin/cytoskeletal interaction resulting in modulation of directionality of cellular migration (Pankov et al., 2005). A switch in the integrin subtype has been described as underlying the changes in cellular motility from a random motion to a more directional one. Thus in epithelial cells an integrin switch between $\alpha 5 \beta 3$ to $\alpha 5 \beta 1$ can result in a different behaviour in motility (Danen et al., 2005; White et al., 2007). Nox1 was demonstrated to be important in this process wherein the knockdown of Nox1 lead to a loss of directional migration suggesting that Nox1 could be a switch between random and directional migration.

Interestingly the persistence in the Nox2^{-/-} BMM also increased following AngII stimulation. The data would imply that downstream signaling of AngII via Nox2 could possibly be inhibiting the increase in persistence, thus the absence of Nox2 had resulted in a larger increase in cellular persistence. Alternatively, other non-Nox2 dependent signalling

pathways, such Nox4, may be stimulated underlying the increase similar to the increase in speed of migration. Further experiments would be required to determine this however if this explanation is established then it would demonstrate that AngII stimulation of Nox4, or other non-Nox2 signalling pathway, is an alternative means by which BMM can increase their persistence of migration towards CSF-1 stimulation.

4.4.5 SUMMARY

Taken together the data suggests that AngII has a slowing effect on the CSF-1 induced chemotaxis in BMM. The influence of AngII on the acute responses of CSF-1 is Nox2 dependent. The possibility exists that some of the AngII effect occur via Nox4. These aberrations in cellular migration towards a chemotactic stimulation in Nox2^{-/-} BMM could, in part, play a role in the reduced fibrosis and hypertrophy observed.

However, these experiments have been conducted in vitro. The combined influence will greatly differ in vivo where the cells are exposed to AngII for a longer duration and the cells attracted by different chemotactic and risk factors. As such oleic acid and AngII both increased VSMC migration but co-incubation had an additive effect (Greene et al., 2001). In the next chapter we investigate in-vivo the cell specific role of Nox2 in circulating inflammatory and resident cardiac cells in AngII induced cardiac fibrosis.

5 CELL SPECIFIC ROLE OF NOX2 IN ANGIO II INDUCED CARDIAC HYPERTROPHY AND FIBROSIS

5.1 INTRODUCTION

5.1.1 INVOLVEMENT OF NOX2 IN CARDIAC FIBROSIS AND HYPERTROPHY

The role of Nox2 in cardiac hypertrophy and cardiac fibrosis has been demonstrated in many studies in the literature. Bendall et al (Bendall et al., 2002a) have shown that left ventricular hypertrophy (LVH) induced by a subpressor dose of AngII infusion (0.3mg/kg/day) was inhibited in Nox2^{-/-} mice. As Nox2^{-/-} mice have a lower baseline blood pressure than WT mice it was theoretically possible that the reduced hypertrophy could be a result of this. In order to exclude this possibility Byrne et al (Byrne et al., 2003) investigated the effect of a pressor dose of AngII (1.1 mg/kg/day) on cardiac hypertrophy. This research demonstrated that a modest pressor dose of AngII, which raised the blood pressure in Nox2^{-/-} mice to a similar level to that in WT mice, still failed to induce significant hypertrophy in the Nox2^{-/-} mice. This finding supported the conclusion that Nox2 was essential for the development of AngII induced cardiac hypertrophy.

The role of Nox2 in AngII induced cardiac fibrosis has been investigated by Johar et al (Johar et al., 2006). In WT mice, subcutaneous infusion of a pressor dose (1.1mg/kg/min) of AngII for 2 weeks significantly increased NADPH oxidase activity. In line with this, the level of interstitial fibrosis as measured by the mRNA expression of fibronectin, procollagen I, and connective tissue growth factor was also significantly increased. These effects were all inhibited in Nox2^{-/-} hearts.

These studies demonstrate an important role for Nox2 in the development of AngII dependant cardiac hypertrophy and fibrosis. However as these experiments had been undertaken in Nox2^{-/-} mice in which the Nox2 had been knocked out globally it was not possible from these studies to ascertain in which cell the loss of Nox2 was critical. In order to explore this the following experiments use chimeric mice to investigate the role of Nox2 in peripheral circulating inflammatory cells and resident cardiac cells in the development of AngII induced cardiac fibrosis and hypertrophy.

Chimeric mice are established by the replacement of bone marrow cells in the recipient mice of a particular genotype with the bone marrow cells from mice of a different genotype. This technology is a crucial technique in cardiovascular and other research work which enables disease processes to be investigated (Herijgers et al., 1997; Schiller et al., 2001; Van et al., 1997; Van et al., 2000). In the current experiments the formation of chimeric mice resulted in WT mice with circulating bone marrow cells which are deficient in Nox2. Thus this enabled the contribution of Nox2 in circulating cells, towards the development of cardiac fibrosis and hypertrophy following AngII stimulation, to be investigated.

5.1.2 ANG II MODULATION OF HAEMATOPOIESIS

The bone marrow RAS and in particular its effector hormone, AngII, modulates the control of haematopoiesis by influencing a variety of differentiation and maturation factors (Haznedaroglu, 1999; Weber et al., 1999). As such, hypertensive patients treated with losartan experienced mild normocytic anaemia after renal transplantation (del et al., 1998). In keeping with these observations, Mrug *et al* (Mrug et al., 1997) showed that Ang II

increased the growth of erythroid progenitors *in vitro*, an effect completely abolished by losartan. Further evidence for the role of AngII modulation in haematopoiesis comes from studies which demonstrate that AngII infusion increased the haematocrit in ACE knockout mice (Cole et al., 2000). Thus taken together these findings support a role for AngII in the modulation of bone marrow hematopoiesis.

The mature blood cells, derived from the bone marrow, undergo a complex series of proliferative and differentiation steps. During this process there is a changing array of adhesion molecule expression in these progenitor cells. These markers help to distinguish and identify both lineage and maturational stage of the cells and therefore reflect on their function.

The antigenic differentiation of two monocyte subsets in mice was first achieved on the basis that monocytes could be subdivided according to their expression of chemokine receptor 2 (CCR2), L-selectin (CD62L) and CX₃C-chemokine receptor 1 (CX₃CR1). One monocyte subset expressed CCR2, CD62L and only moderate amounts of CX₃CR1 (CCR2⁺ CD62L⁺ CX₃CR1⁻) whereas the second did not express CCR2 or CD62L but expressed higher amounts of CX₃CR1 (CCR2⁻ CD62L⁻ CX₃CR1⁺). The CCR2⁺ monocyte subset, as expected, migrated towards the CCR2 ligand, CC-chemokine ligand 2 (CCL2; also known as MCP1) (Palframan et al., 2001). The expression of CCR2 and the capacity to migrate towards CCL2 is consistent with the important role of this chemokine and its receptor in the recruitment of monocytes to inflammatory lesions, and therefore the murine CCR2⁺ monocytes are known as the 'inflammatory' subset (Gu et al., 1998; Kurihara et al., 1997; Kuziel et al., 1997; Lu et al., 1998).

The receptors that are expressed by the inflammatory-monocyte subset are broadly considered to be chemokine and adhesion receptors, involved in the recruitment of leukocytes to an inflammatory lesion. In addition, Geissmann et al (Geissmann et al., 2003) identified Ly6C (which is part of the epitope of Granulocyte Receptor 1) as an additional marker of CCR2⁺ monocytes in mice. These studies indicated that CCR2⁺CD62L⁺CX₃CR1^{low}Ly6C⁺ mouse monocytes correspond to CD14^{hi}CD16⁻ (classic) human monocytes, which are also CCR2⁺CX₃CR1^{low} and that CCR2⁻CD62L⁻CX₃CR1^{hi}Ly6C⁻ mouse monocytes correspond to CD14⁺CD16⁺ human monocytes, which also express large amounts of CX₃CR1. Thus this analogy allowed the results in mice to be correlated with humans as summarized in the table below.

MICE	HUMANS
CCR2 ⁺ CD62L ⁺ CX ₃ CR1 ^{low} Ly6C ⁺	CD14 ^{hi} CD16 ⁻
CCR2 ⁻ CD62L ⁻ CX ₃ CR1 ^{hi} Ly6C ⁻	CD14 ⁺ CD16 ⁺

The evolving evidence from the literature would suggest that bone marrow-derived monocytes with the phenotype CCR2⁺CX₃CR1^{low}Ly6C⁺ are released into the circulation (Figure 5.1). In the presence of inflammation, the bone-marrow-derived monocytes can respond to pro-inflammatory cues, migrate into inflamed tissues and differentiate into macrophages (Geissmann et al., 2003; Qu et al., 2004). These Ly6C⁺ monocytes are immature cells and are thought to mature to Ly6C⁻ cells in the circulation (Sunderkotter et al., 2004). In the absence of inflammation, a switch in monocyte phenotype occurs, to tissue-resident macrophages and dendritic cell, the mechanism of this regulation is unknown. This then generates monocytes that are

postulated to enter the tissues and replenish the tissue population of macrophages and dendritic cells. These are known as the ‘resident’ monocyte population with the phenotype CCR2-CX₃CR1^{hi}Ly6C- (Geissmann et al., 2003).

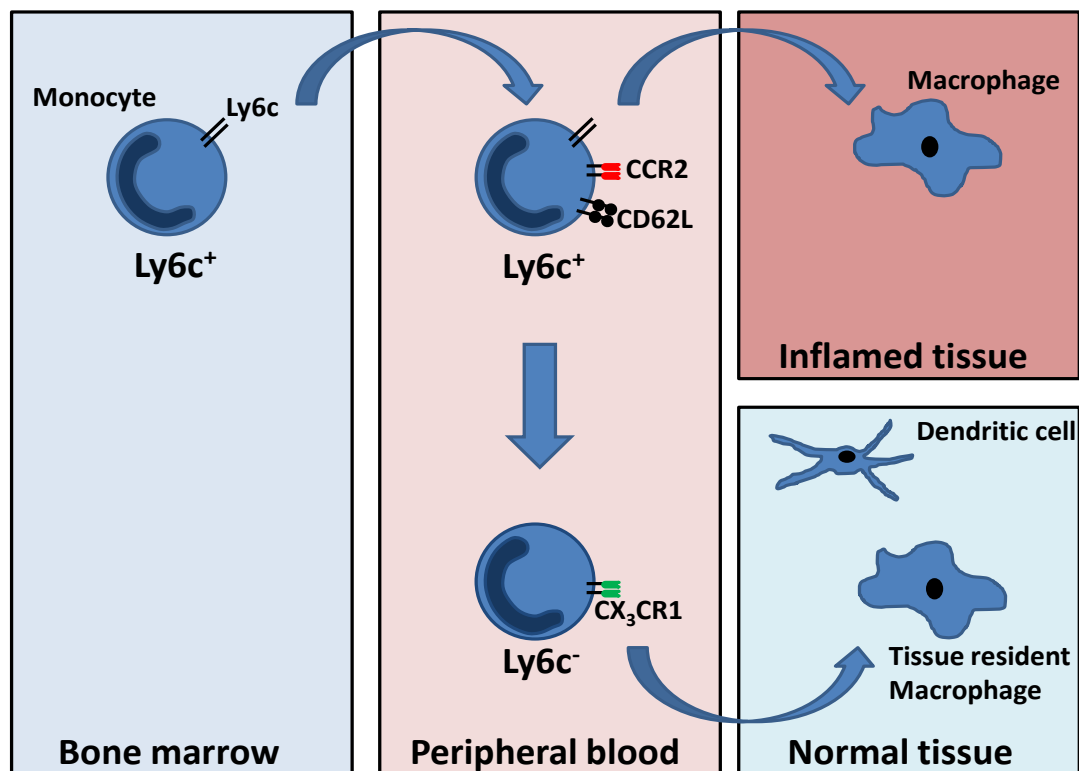


Figure 5-1 Maturation of monocyte subsets in mice.

Ly6C⁺ bone-marrow monocytes are released into the peripheral blood. Ly6C⁺ monocytes are thought to respond to pro-inflammatory cues (such as CCL2), and be recruited to inflammatory tissue. Most ‘inflammatory’ monocytes are thought to differentiate into macrophages, which are important for clearance of pathogens and for the resolution of inflammation. In the absence of inflammation, CX₃CR1^{hi}Ly6C⁻ monocytes enter the tissues and replenish the tissue-resident macrophage and dendritic cell populations.

Thus Ly6C^{hi} and Ly6C^{low} expressing monocytes show differing functions in normal physiological processes. The infarcted heart sequentially and actively recruits Ly6C^{hi} and Ly6C^{low} expressing monocytes via CCR2 and CX₃CR1, respectively. Ly6C^{hi} expressing monocytes dominate the early phase of recruitment and exhibit phagocytic, proteolytic, and inflammatory functions. Ly6C^{low} expressing monocytes dominate the later phase and have attenuated inflammatory properties, and express vascular-endothelial growth factor. Consequently, Ly6C^{hi} expressing monocytes digest damaged tissue, whereas Ly6C^{low} expressing monocytes promote healing via myofibroblast accumulation, angiogenesis, and deposition of collagen (Nahrendorf et al., 2007). However, the numbers of these different types of monocytes can alter in disease processes. For example in hypercholestromia the numbers of Ly6C^{hi} expressing monocytes are increased (Libby et al., 2008; Swirski et al., 2007). Also myocardial infarction, in atherosclerotic mice with chronic Ly6C^{hi} monocytosis, results in impaired healing, underscoring the need for a balance between the numbers of Ly6C^{hi} and Ly6C^{low} expressing monocytes (Panizzi et al., 2010).

The change to the numbers of Ly6C^{hi} expressing monocytes in hypertension has not been investigated. One of the aims of this chapter is to characterise the change in the number of Ly6C^{hi} expressing monocytes in the bone marrow and peripheral blood of WT and Nox2^{-/-} mice following AngII induced hypertension.

5.1.3 ADHESION AND MIGRATION OF CIRCULATING MONOCYTES

Leukocyte recruitment to the arterial wall and the heart is a hallmark of the early stages of hypertension and hypertensive heart disease in which

AngII is known to play a critical role (Hernandez-Presa et al., 1997; Mervaala et al., 1999). AngII is capable of promoting monocyte adhesion and activation *in vitro* (Hahn et al., 1994) and as such monocyte migration into the vessel wall, a critical event leading to the development of cardiac fibrosis, can be attenuated by ACE inhibition or by pretreatment with an AT₁ receptor antagonist (Hahn et al., 1994; Hernandez-Presa et al., 1997; Mervaala et al., 1999). Moreover, AngII superfusion of the rat mesenteric microcirculation induces leukocyte adhesion in venules (Piqueras et al., 2000). Therefore, AngII may be a key factor in the leukocyte adhesion and subsequent subendothelial infiltration observed in hypertension.

The interaction between the circulating inflammatory leukocytes and the inflamed endothelium is mediated by three groups of adhesion molecules namely the integrins, selectins and the Ig superfamily (Carlos et al., 1990; Carlos and Harlan, 1994). The initial low affinity interaction between the leukocytes and the endothelium, manifesting itself as rolling behavior, involves the participation of selectins. The rolling leukocyte by the participation of integrins and Ig superfamily becomes firmly adherent on the vessel wall which allows the process of transendothelial leukocyte migration to occur. Additional steps in this process such as slow rolling, adhesion strengthening and spreading, intravascular crawling and the route of transmigration makes this process even more complex (Ley et al., 2007).

The selectins, which mediate leukocyte rolling, are a family of lectin-like molecules of which 3 types namely L-selectin, P-selectin and E-selectin have been described. The P-Selectins are expressed on endothelial cells and platelets whereas E-selectins are only expressed on endothelial cells. In contrast L-selectins are only expressed on leukocytes. It is widely accepted

that the L-selectin shedding is involved in the recruitment by leukocyte extravasation into inflamed tissues (Kishimoto et al., 1989). The role of AngII in this regulation of L-selectin shedding has been demonstrated from different lines of evidence. Clinical studies have shown that L-selectin density on lymphocytes and monocytes/macrophages was significantly lower in hypertensive patients than in normotensives (Mills et al., 2002; Zapolska-Downar et al., 2006). In an *in vitro* study, Vega et al (Vega et al., 2010) demonstrated that AngII, in a dose dependent manner, induced shedding of L-selectin in human neutrophils rapidly over the course of minutes reaching maximum shedding at five minutes. In keeping with this Prasad et al (Prasad et al., 2001) demonstrated a significant increase in L-selectin expression on monocytes, lymphocytes and granulocytes in patients treated with losartan, an AngII receptor antagonist. Thus an AngII dependent mechanism might lead to direct L-selectin shedding and leukocyte extravasation into local inflammatory sites in hypertensive cardiac disease.

There is also evidence supporting the involvement of ROS in the initial adhesion process involving L-selectin. In neutrophils, H₂O₂ exposure increases their adhesion to the endothelium (Fraticelli et al., 1996). Also metalloprotease 17 (or ADAM 17), a sheddase of L-Selectin promoting shedding, has been shown to be redox sensitive (Wang et al., 2009b). Therefore AngII may mediate its cardiac effect on L-selectin expression by mediating the production of ROS, in particular from Nox2. Thus this could explain the lack of cardiac fibrosis and hypertrophy in Nox2^{-/-} mice following AngII stimulation.

The leukocytes adherent to the vessel wall are required to mobilize themselves to the nearest junctional site on the endothelium before starting

diapedesis (Schenkel et al., 2004). Diapedesis is a rapid process in which the leukocytes extend themselves by the formation of pseudopods, across the endothelial border. This requires the rapid disassembly and reassembly of the cytoskeleton enabling the cell to change shape. The process of diapedesis has been reviewed in detail in the literature (Kamei and Carman, 2010; Muller, 2003) and the redox sensitive regulation of the actin cytoskeleton as been described in chapter 8. Thus AngII, via ROS from Nox2, could influence the dynamics of the actin cytoskeleton and therefore the ability of the leukocytes to diapedese. Again this could account for the lack of cardiac fibrosis and hypertrophy observed in Nox2^{-/-} mice following AngII stimulation.

Thus another aim of this chapter is to investigate the expression of L-selectin and filamentous actin in monocytes derived from the bone marrow and peripheral blood of WT and Nox2^{-/-} mice following AngII stimulation.

5.1.4 AIM

This chapter aims to successfully establish chimeric mice which will be used to investigate the cell specific role of Nox2, namely that of resident cardiac cells versus circulation inflammatory cells, in AngII induced cardiac fibrosis and hypertrophy.

In WT and Nox2^{-/-} mice stimulated with AngII the numbers of inflammatory, Ly6C^{hi} expressing bone marrow and blood monocytes and their expression of L-Selectin and filamentous actin will also be investigated.

5.2 METHOD

5.2.1 *GENERATION OF CHIMERA MICE*

Mice aged 8-12wks were used as recipient mice. The mice were initially irradiated with whole body irradiation. The purpose of the irradiation is to completely deplete the haematopoietic system of the recipient mice to allow the repopulation of the bone marrow cells from the donor mice. The ionizing radiation causes lesions in cellular DNA (Duran-Struuck and Dysko, 2009; Prise et al., 2005). Due to the large number of lesions, many of them are not repaired or suffer misrepair by the DNA repair system that can lead to apoptosis (Prise et al., 2005). The survival rates of the mice tend to be lower if they are irradiated with a single high dose (9.5Gy) of radiation rather than two doses of 6Gy (Cui et al., 2002). At our institute the practice is to irradiate the mice with a single dose of 7Gy of radiation in a linear accelerator. The marrow from the donor mice is harvested the following day for injection into the recipient mice by tail vein injection.

It is important to realize that alternative methodologies exist to BMT chimeric mice. In most cases it is technically feasible to carry out similar experiments by making use of transgenic technology. However, the setting up of BMT chimeric mice is usually faster and more cost effective. In BMT experiments the cells given to the recipient mice are histocompatible as the mice are genetically identical due to their inbreeding thus preventing any graft versus host disease. Crucially the acute replacement of bone marrow in the BMT chimeric mice prevents for any chronic compensatory changes to take effect as could happen in transgenic mice. However, a limitation is that irradiation itself could unknowingly have had other effects.

5.2.2 PREPARATION OF MARROW FOR INJECTION

The marrow was obtained following culling of the mice with terminal anaesthesia and harvesting their femurs for flushing. The mice used in the experiments were of similar age to the recipient mice as it has been shown that the age of the donor mice does not affect the repopulation efficiency of the bone marrow (Harrison and Astle, 1982). The murine femurs are cleaned of the surrounding tissue by gentle pulling using forceps to prevent contamination of the bone marrow cell on flushing of the marrow. The procedure was undertaken in a tissue culture hood so as to maintain sterility. Once the bone had been cleaned a 21-gauge needle was used to thread through both ends of the bone. Then, using a 5-mL syringe with a 21G needle, the bone marrow was flushed out of the bone with 5 mL of serum free DMEM into a petri dish. An additional 5 mL of serum free DMEM was added to the petri dish, the cells collected and placed into a falcon tube and centrifuged for 5 min at 1000g at room temperature. The supernatant was discarded and the pellet resuspended in serum free DMEM. Half a milliliter of serum free DMEM was used to resuspend the pellet for every mouse culled and 0.25ml of suspension was then used to inject into each mouse. Thus the marrow from one mouse was sufficient to inject two recipient mice.

5.2.3 TAIL VEIN INJECTION

Before the tail vein injection the mice were heated in an incubator at 30°C to dilate the tail veins. The mice were then held in a restraining device to immobilise them and allow access to the mouse tail. The vein was identified visually, as normally the mice have a vein coursing both the lateral sides of the tail which can be used for injection, and 0.25mL of marrow

suspension was carefully injected into the tail vein by a slow continuous push.

Each run had a control mouse which did not receive marrow injection. These mice served as controls for the lethal irradiation dose that the mice received. Hence in the absence of any BMT approximately two weeks after the irradiation the control mice were culled due to their declining health.

Four days after administration of the bone marrow the cellular repopulation starts through the homing of the cells to the bone marrow cavity (de Winther and Heeringa, 2003). By the seventh day after the transplantation, the bone marrow repopulation is completely restored (de Winther and Heeringa, 2003). The circulating leukocytes are replaced with donor derived cells between 2-4 weeks after the transplantation. Therefore a period of one month was allowed for the recovery of the bone marrow and peripheral blood cells before any experimentation. The repopulation of the circulating blood cells was confirmed by FACS analysis of peripheral blood via tail vein blood sampling and ultimately by genotyping of the marrow cells.

5.2.4 FACS COUNTING OF CELLS IN TAIL VEIN BLOOD

To confirm the repopulation of the marrow and the peripheral blood count, tail vein blood samples were taken every 4 days over 16 days. For this the mice were anaesthetized (2.5% isoflurane(Abbott, UK)//97.5% oxygen) and the tail tip excised with scissors. Five drops of blood was allowed to drop into an eppendorf tube containing EDTA. The tail was cauterized to achieve haemostasis and the mouse returned to its cage.

One ml of RBC lysis solution (9 parts NH_4Cl (0.16M) and 1 part Tris-HCl (0.17M;pH7.6) pH adjusted to 7.6 with HCl) was added to the eppendorf

containing the blood and left to stand for 10mins. The eppendorf was then centrifuged at 1200rpm for 5mins. The supernatant was discarded and the pellet resuspended in PBS and moved to a FACS tube. The cells were incubated for 1hr on ice with antibody against CD45 (MACS 1:200) [Miltenyi Biotec], a pan-leukocyte marker. The sample was then centrifuged, resuspended in PBS and analysed by FACS. The machine was set to count the number of events over 10mins.

5.2.5 *MARROW CELLS FOR GENOTYPING*

Genotyping (undertaken with Simon Walker) was used to confirm the presence or absence of the Nox2 gene from the marrow cells of the chimera mice. The marrow was harvested and digested in a solution of Proteinase K ((10mg/ml) Roche, UK) in an alkaline buffer (0.1 M Tris EDTA, 0.1 M Na₂EDTA, 1% SDS and 0.15M NaCl, pH 8.0) for 2-4 hours at 55°C. cDNA was precipitated in isopropanol, washed with 80% ethanol and resuspended in TE buffer (1mM EDTA, 10 mM Tris, pH 8). Amplification of resultant cDNA was conducted in a thermal cycler (AB Applied Biosystems, UK) involving 35 cycles of denaturing (30 seconds at 94°C), annealing (30seconds at 56°C) and synthesis (30 seconds at 72°C). A duplex reaction on 1µl of cDNA was achieved using primers (10µM) for Nox2 in a 25µL total reaction volume containing 12.5 µL Red Taq Ready Mix, 0.5 µL forward primer, 0.5µL reverse primer 1, 1 µL reverse primer 2 (Table 5.1) and 9.5 µL of nuclease-free H₂O. Subsequent polymerase chain reaction (PCR) products were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide (400 ng/mL) in 40 mM Tris-acetate, 1 mM EDTA and visualized under ultraviolet

light. A single band at ~240 basepairs (bp) confirmed WT status and a single band at ~195bp confirmed a Nox2^{-/-}.

Table 5-1 Primers used in genotyping

Primer	Sequence
Forward	AAG AGA AAC TCC TCT GCT GTG AA
Reverse 1	CGC ACT GGA ACC CCT AGA AAA GG
Reverse 2	GTT CTA ATT CGA TCA GAA GCT TAT CG

5.2.6 OSMOTIC PUMP INSERTION

Osmotic pumps (Figure 10.2) were used to administer AngII (0.3mg

or 1.1mg/kg/day) *in vivo* over 14

days. Pumps were inserted into WT,

Nox2^{-/-} , WT chimeric and Nox2^{-/-}

chimeric mice These pumps are

designed with an inner reservoir

(bladder) to hold the drug

surrounded by a salt sleeve and a

semi-permeable outer capsule. The

high osmolality of the salt sleeve

causes water to flux across the

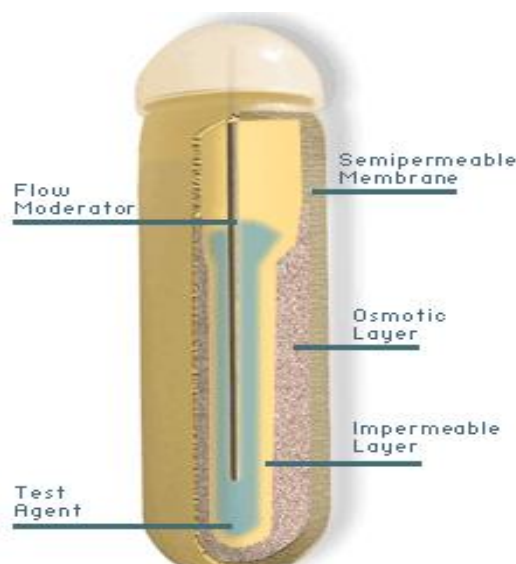


Figure 5-2 Alzet Corp® Model 2001

capsule into the pump, which compresses the flexible reservoir dispensing the drug at a set rate via a flow moderator. The permeability of the outer capsule controls the flux of water and thus the amount of drug displaced irrespective of the drug concentration or formulation, for example, pump

model 2001 which was used in the current series of experiments, infuses at a constant rate of 0.25 μ l/h over 14-days. Equivalent control groups were implanted with osmotic pumps containing saline.

To implant the osmotic pumps the mice were anaesthetized (2.5% isoflurane (Abbott, UK)//97.5% oxygen) and an area shaved on their upper back. The site was sterilized with iodine before making a subcapular incision (1-2cm). A subcutaneous pocket was created by blunt dissection and the osmotic pump was then implanted into this space with the flow moderator facing downwards into a posterior subcutaneous pocket created. The incision was closed with 5/0 vicryl suture. Buprenorphine (Vetergesic™, UK; 0.05mg/kg) analgesic was administered by intramuscular injection at the start of the procedure and the mice were allowed to recover in a warmed (37°C) cage for a minimum of 30 minutes.

Infusion of pharmacological agents such as AngII via implanting osmotic pumps subcutaneously is well-established. The AngII induces hypertension and cardiac hypertrophy (Berry et al., 2001) as well as causing a proinflammatory phenotype (Chen et al., 1998) resulting in cardiac fibrosis, therefore mimicking the development of LVH in humans (Berry et al., 2001; Weber and Brilla, 1991). Infusing AngII at a low dose (0.3mg/kg/day) over a 2-week period causes a small degree of hypertrophy without significantly increasing blood pressure. However, hypertension can be achieved with higher pressor doses of AngII (>0.7mg/kg/day) (Kawada et al., 2002) which is accompanied by a greater hypertrophy and increased cardiac fibrosis (Johnson et al., 1992; Sun et al., 1993; Sun et al., 1997). A criticism of this model is that plasma levels of AngII can be substantially

greater in some animals (up to 80 fold higher in canines) than those seen in humans (Kawada et al., 2002; Weiss et al., 2001).

5.2.7 NON-INVASIVE ASSESSMENT OF BLOOD PRESSURE MEASUREMENT

Measurement of systolic blood pressure is an essential component of overall haemodynamic analysis (Kurtz et al., 2005a). Non-invasive blood pressure measurement was conducted by tail-cuff plethysmography (Kent Scientific, USA). Tail cuff plethysmography is an inexpensive, relatively simple method of assessing systolic blood pressure. However, there are several limitations to this method the predominant one being that, in order to obtain measurements the mice have to be restrained which may cause stress thus altering blood pressure, with differences in stress levels between mice or within the same mouse over subsequent days will add to the variations in the assessment of blood pressure. The mice are exposed to further stress by exposing the mice to the heated environment (ambient temperature of 25°C), which is required to ensure sufficient dilation of the tail artery to provide a clear signal.

It is critical that the mice are trained for several days before the start of the experimental protocol such that they become accustomed to the restrainer. As such prior to the assessment of blood pressure, each mouse underwent a series of training sessions over a five-day period which consisted of introducing each mouse into a plastic tube restrainer, and positioning both the occlusion and plethysmography cuffs over the tail, prior to cuff inflation. The duration of time in the holder and number of inflations were gradually increased over the training sessions to acclimatise the mice to the procedure. Once acclimatised, an average blood pressure measurement was taken from

six consistent readings, per session per mouse, acquired by Chart® software (Adinstruments, UK) via a Powerlab® amplifier (Adinstruments, UK).

The acclimatization should reduce some of the variation in measuring the blood pressure. Nevertheless, blood pressure naturally varies even over short periods of time and plethysmographically derived blood pressure is only a snap shot (~15minutes) of a mouse's systolic blood pressure during the day, when this normally nocturnal animal is less active. Therefore, the limited number of measurements that can be obtained may not reflect the true average blood pressure. However it is generally considered that although tail cuff plethysmography has its limitations it can be used to confidently assess the substantial changes in blood pressure expected with interventions such as AngII infusion (Kurtz et al., 2005b). Thus the blood pressure measurements were undertaken at the same time everyday.

5.2.8 ORGAN, MARROW AND BLOOD HARVEST

Following the period of AngII infusion blood and organs were harvested from the mice. For this mice were anaesthetised using Isoflurane (2-2.5% isoflurane/oxygen) and a thorocotomy performed to allow access to the inferior vena cava. The inferior vena cava was then pierced using a 29G needle and 0.5-1 mls of blood was collected into EDTA treated tubes. The femurs were also harvested for the later marrow harvest as described earlier in section 5.2.2.

For histological analysis the heart must be harvested in diastole. To achieve this a KCl (5M)-PBS solution was infused into the LV, via the already performed thoracotomy, at an approximate pressure of 115mmHg for 15-sec. The right-atrium was cut to allow the blood and solution to flow. The heart

was then harvested, rinsed in cold PBS (4°C), any connective or adipose tissue removed, blotted dry and weighed (Sartorius, UK). The heart was then dissected into the LV, right ventricle (RV) and atria and the individual sections weighed. For histological studies the LV was divided into three transverse sections, a small section of ~1mm at the base of the heart and a larger section (4-6mm) at the apex were frozen, whilst a middle section approximately between 2-3mm was preserved for histological studies in 1% paraformaldehyde and stored at 4°C. Tissue for frozen sections were snap frozen in liquid nitrogen and stored in vapour phase of liquid nitrogen (-150°C) until required.

5.2.9 HISTOLOGICAL PREPARATION

The hearts for histological studies were arrested in diastole prior to harvesting. The tissue was preserved for paraffin sectioning by fixing in paraformaldehyde (1% in PBS at 4°C) for up to 12 hours. Following this the tissue was washed in 2 changes of PBS for 30 minutes at 4°C and then subsequently moved into 80% ethanol (in PBS at 4°C). Samples were set in paraffin blocks after a series of automated dehydration steps overnight (Shandon Hypercentre XP; 70% ethanol room temperature (RT), 1h; 96% ethanol RT, 1h; 96% Ethanol, RT, 1h; 100% ethanol, RT, 2h; 100% ethanol, RT, 2h; 50% Xylene+50% ethanol (100%), RT, 2 h; Xylene (100%), RT, 2h; Xylene(100%), RT 2h; Paraffin, 60°C, 1.5h; Paraffin, 60°C, 1.5h). Sections were subsequently cut using a Microtome (Micron HMS60, Germany) generally at 6µm on Superfrost plus slides (VWR, UK).

5.2.10 *PMA STIMULATED ROS PRODUCTION IN MARROW CELLS: DHE*

The process of setting up the BMT chimeric mice could have altered the ROS response within the marrow cells. Thus to investigate the ROS response of the marrow cells from chimeric mice Phorbol 12-myristate 13-acetate (PMA) stimulation was undertaken. PMA is a diacylglycerol analogue which stimulates protein kinase C (PKC) which in turn phosphorylates p47^{phox} and stimulates Rac resulting in Nox2 activation. The cells were placed in FACS tubes, RBC lysis solution was added and the cells left to stand for 10mins. The tubes were centrifuged at 1200rpm for 5mins. The supernatant was discarded and the pellet resuspended in PBS and moved to a FACS tube. Dihydroethidium (DHE) (Sigma) was added to a final concentration of 10µM. DHE is oxidised by ROS to ethidium bromide which can intercalate with DNA and fluoresce enabling FACS analysis. Thus DHE assay is a means of measuring the intracellular ROS production. To the appropriate tubes PMA stimulation was added to a final concentration of 100ng/ml to stimulate ROS production. The tubes are left to stand at 37°C for 10 minutes, centrifuged at 1200rpm for 5 minutes following which the cells washed in cold PBS with 5% FBS. ROS production following PMA stimulation in the marrow cells was quantified by FACS.

5.2.11 *PMA STIMULATED ROS PRODUCTION IN MARROW CELLS: LUCIGENIN*

Measurement of ROS response was also undertaken by lucigenin which is a well established method in our laboratory and has been described in chapter 2. To summarise the marrow cells were placed in FACS tubes, RBC lysis solution was added and the cells left to stand for 10mins. The tubes were

centrifuged at 1200rpm for 5mins. The supernatant was discarded and the pellet resuspended in PBS. Samples were seeded (1000cells/well) on 96-well all white microplate in 75 μ l of intracellular solution (ICS; 140mM KCl, 1mM EGTA, 6 mM glucose, 20 mM K-PIPS, 1 mM MgCl₂, pH 7) in the presence or absence of PMA (10 μ M) stimulation. Cells were permeabilised with 2 μ L of 0.5% saponin to allow NADPH (300 μ M) and lucigenin into the cell.

5.2.12 *MONOCYTE SEPARATION FROM BONE MARROW AND BLOOD*

The bone marrow and the blood samples were made up to 5ml with 5% PBS and then rolled down the side of 10 ml falcon tube containing 5 mls of warm Histopaque Ficol 1038 (Sigma) so that the sample formed a separate layer on top of the Ficol. The sample was then centrifuged at 2000rpm for 30mins at room temperature to separate it into layers. The white layer, formed at the interface between the Ficol and the sample, comprised of mononuclear cells. The pellet consisted of RBC and polymorphonuclear cells. The mononuclear layer included monocytes and lymphocytes. This layer was carefully removed by suction with a pipette and placed into a new falcon tube containing 5mls of 5%PBS. The sample was then spun at 1200rpm for 5mins and the pellet resuspended in 1mls of 5% PBS and counted. The cells were then mixed in an eppendorf tube with CD11b Microbeads (Miltenyi Biotec) such that 10 μ l of microbead was mixed with 90 μ l of cells, containing a maximum of 10⁷ cells. The CD11b Microbeads were developed for the separation of mouse cells based on the expression of the CD11b antigen (integrin α_m chain). The CD11b antibody reacts with the 170 kDa α_m subunit of the CD11b/CD18 heterodimer ($\alpha_m\beta_2$ integrin). This integrin functions as a receptor for complement (C3bi), fibrinogen or clotting factor X. The cells

were mixed with the microbeads in at 4°C for 30mins. The monocytes have high expression of CD11b (Sunderkotter et al., 2004) as compared to lymphocytes and were therefore selectively separated out by passing the sample through a MACS Column where the beads were attracted to a magnet. The cells not attached to a CD11b-microbead were washed out the bottom of the column. The column was washed three times and then the cells attached to the beads i.e. CD11b+ cells were obtained by washing the column without the magnet. This last wash was collected into a new falcon tube for FACS analysis.

5.2.13 FACS ANALYSIS OF MONOCYTES

The monocytes separated from the MACS Column were centrifuged, resuspended in 5%PBS and counted on a haemocytometer. One hundred thousand cells were added to FACS tubes and incubated with an antibody against Ly6c (1:10; Miltenyi Biotec), conjugated to FITC and MEL-14 (1:100; Santa Cruz), conjugated to PE against L-selectin was used. After incubation for 30mins the cells were washed and resuspended in 1ml of 5% PBS for quantification by FACS.

To quantify the levels of F-actin the cells were fixed for 10mins with 4% paraformaldehyde, permeabilised using 1% tiron prior to the addition of phalloidin-FITC (1:1000, Sigma) which reacts with the filamentous actin inside the cells. After mixing for 10mins, the cells were washed and resuspended in 1ml of 5%PBS for quantification by FACS.

5.3 RESULTS

5.3.1 *ESTABLISHING CHIMERIC MICE*

Following injection of donor bone marrow into irradiated mice, repopulation of peripheral blood was detectable from Day 12. (Figure 5.3) Peripheral blood samples demonstrated that cell count [counted for CD45 (1:10; Miltenyi Biotec), a pan leukocyte marker] was similar between all the mice before irradiation. After irradiation the counts decreased to almost zero. Introduction of donor marrow cells via tail vein injection resulted in the repopulation of marrow and peripheral blood which was detectable after day 12. Control mice which received no marrow cells started to decline in health and two weeks after irradiation were deemed too ill and had to be culled. The experiment demonstrated that a injection of donor marrow cells was able to successfully repopulate the marrow of irradiated mice with the subsequent recovery of peripheral blood count and ultimately survival.

Chimeric mice were established by the reconstitution of the bone marrow with donor marrow obtained from a mouse of a different genotype. This was confirmed by the genotyping of the marrow cells from the chimeric mice. (Figure 5.3) The genotyping of the marrow from Nox2^{-/-} chimeric mice confirmed that it expressed Nox2 and thus was derived from the WT donor mice. Similarly the marrow from WT chimeric mice expressed no Nox2 and therefore had to originate from Nox2^{-/-} mice. The average weight of WT mice pre procedural was 25.5g (range: 20.7g – 32.2g) and that of Nox2^{-/-} mice 26.2g (range: 23.1g – 29.5g)

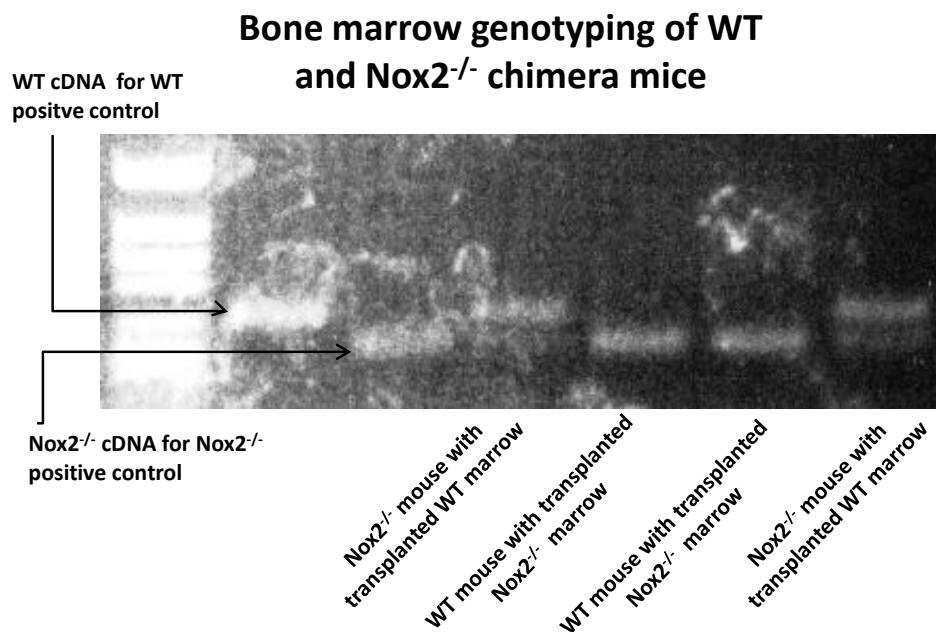
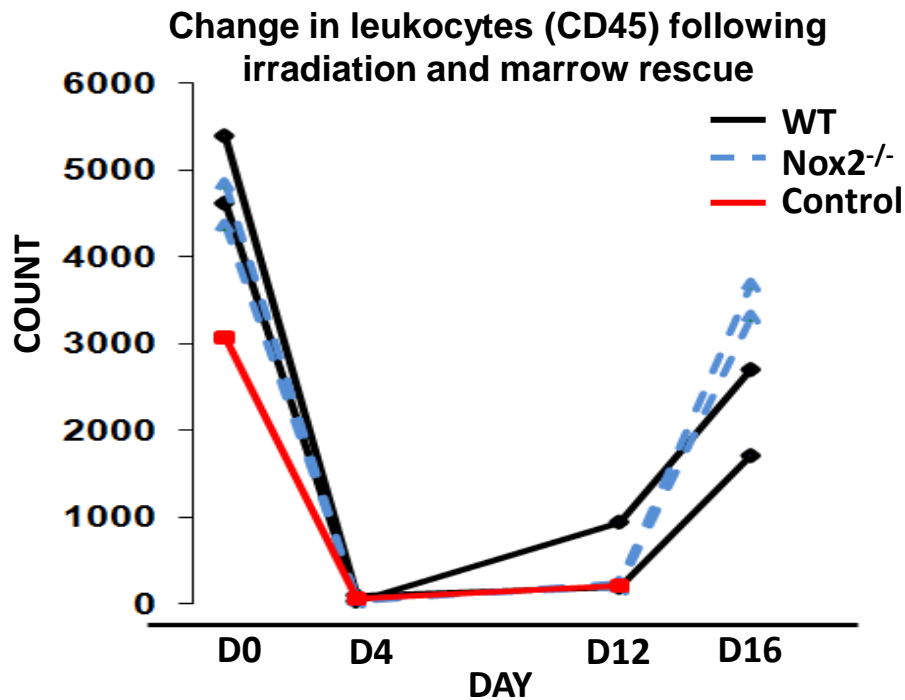


Figure 5-3 The repopulation of peripheral blood cell and marrow genotype of chimeric mice

TOP: The mice re-injected with marrow [two WT (black) and two Nox2^{-/-} (broken blue)] remain healthy in line with the recovery of their peripheral blood cell count over the course of the following 16 days. Mice not injected with marrow cells [one WT (red)] was culled due to poor health.

BOTTOM: Representative blot demonstrating that the genotype of the marrow cells of a Nox2^{-/-} or WT recipient mouse matched those of the donor mouse.

A differential ROS response of the Nox2^{-/-} and WT marrow cells to PMA stimulation would provide an effective functional means to demonstrate that a successful bone marrow repopulation with donor cells retained the characteristics of the donor marrow rather than that of the original bone marrow (Figure 5.4: top). As such, WT marrow cells responded to PMA stimulation with a large ROS response as demonstrated by a shift in the DHE profile to a mean fluorescence value 112.4 units (Figure 5.4, top). However, in the absence of Nox2 this ROS response is lost with a shift in the DHE profile to a mean fluorescence of only 14.7 units. In keeping with this, appropriate ROS response was seen following bone marrow transplantation. Thus a robust PMA response was seen either in Control chimeric WT mice (WT mice transplanted with WT marrow: WT/WT) or chimeric Nox2^{-/-} mice (Nox2^{-/-} mice transplanted with WT marrow: Nox2^{-/-}/WT) i.e. a mean fluorescence shift of 74.9 and 98.2 units respectively. Thus the characteristics of the donor marrow are retained in the recipient mice upon transplantation.

The ROS response of the new repopulated marrow was also assessed with lucigenin (Figure 5.4: bottom). As expected PMA stimulation resulted in a marked increase in the ROS response in marrow cells from WT mice which was not seen in marrow cells from Nox2^{-/-} mice. As such the repopulated marrow from a WT/WT mouse showed a marked ROS response following PMA stimulation. Conversely, the repopulated marrow from a WT/Nox2^{-/-} mouse failed to show a significant ROS response following PMA stimulation. Similarly, the repopulated marrow obtained from a Nox2^{-/-}/WT mice showed a significant ROS response following PMA stimulation consistent with the marrow being that from a WT mouse. Thus the results from both the DHE and lucigenin experiments were consistent suggesting that following

transplantation the repopulated marrow functionally remained that of the donor mouse.

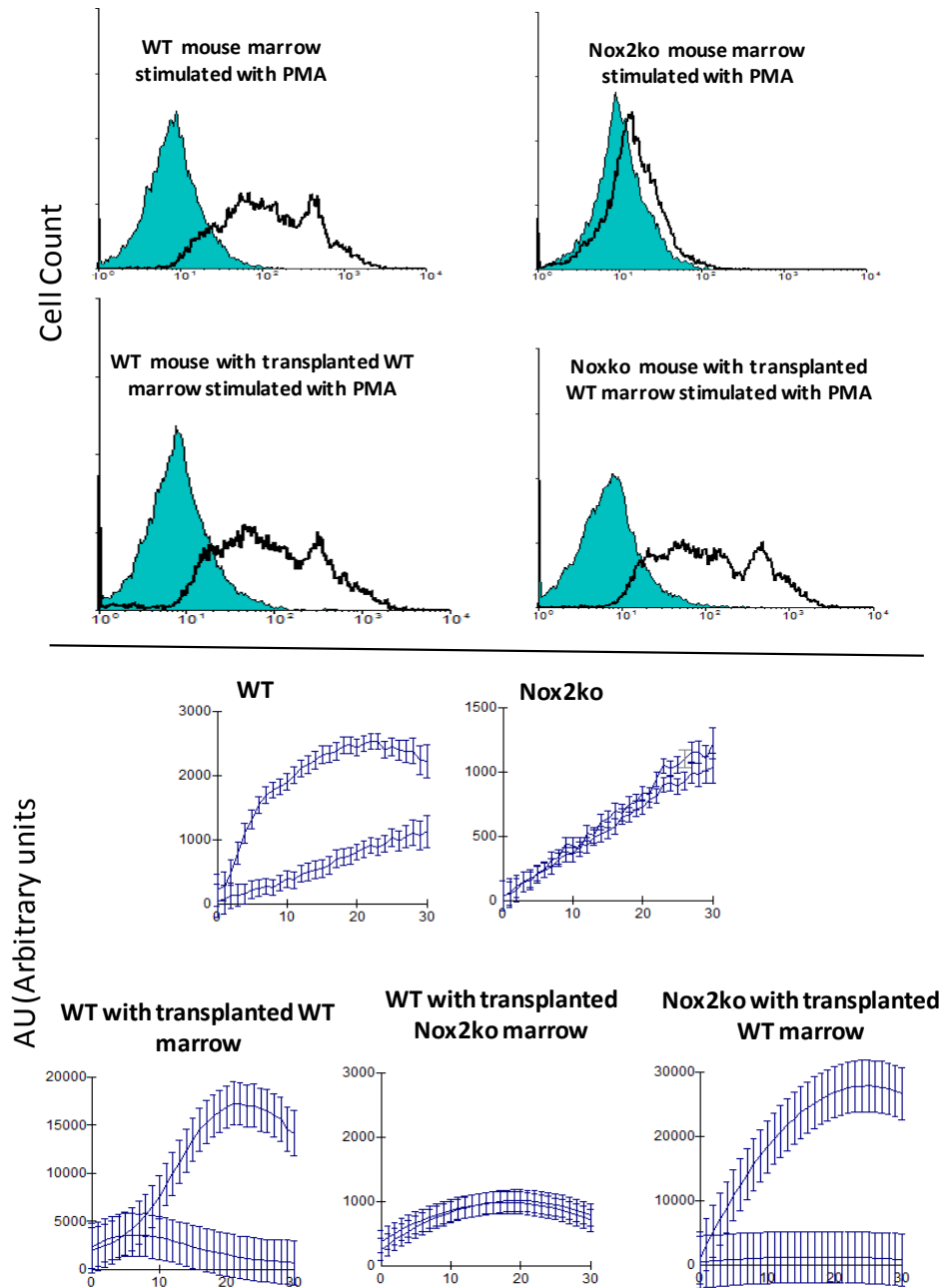


Figure 5-4 ROS response in marrow cells following transplant

Top: ROS response as measured by DHE following PMA stimulation

Following PMA stimulation of WT marrow cells from WT mice (112U), chimeric WT mice with WT marrow (75U) and chimeric Nox2^{-/-} with WT marrow (98U) a

similar response was observed. PMA stimulation of Nox2^{-/-} marrow cells (15U) from Nox2^{-/-} mice showed a reduced ROS response following PMA stimulation.

Bottom: ROS response as measured by lucigenin following PMA stimulation

Following PMA stimulation ROS response measurement by lucigenin demonstrated similar results. Namely WT marrow from WT mice, chimeric WT mice with WT marrow and chimeric Nox2^{-/-} mice with WT marrow demonstrated a large ROS response. Expectedly PMA stimulation of Nox2^{-/-} marrow from Nox2^{-/-} mice or chimeric WT mice with Nox2^{-/-} marrow did not show any measurable ROS response.

Thus the results from both the DHE and lucigenin experiments were consistent suggesting that following transplantation the repopulated marrow functionally remained that of the donor mouse.

Taken together these results suggest that the process of irradiation and subsequent repopulation of the marrow and peripheral blood cells successfully generated viable chimeric mice. The marrow cell by genotype and PMA responsiveness remained that of the donor mouse. Hence the use of these mice would enable the investigation of the cell specific role of Nox2, namely the role of Nox2 in the circulating cells versus that in the resident cells, in the generation of cardiac fibrosis and hypertrophy following AngII stimulation.

5.3.2 CELL-SPECIFIC LOSS OF NOX2 ATTENUATED THE ANGII INDUCED HYPERTENSION

To induce cardiac fibrosis and hypertrophy, AngII pumps were inserted subcutaneously for 2 weeks. During this time, the blood pressure of both the control WT and Nox2^{-/-} mice increased significantly when compared to vehicle infused control (Figure 5.5). The final increase in blood pressure was similar between the WT and Nox2^{-/-} mice. The WT mice blood pressure showed an average increase of 2.5mmHg/day (95%CI:0.58;4.36, p=0.01) similar to that in the Nox2^{-/-} mice of 2.7mmHg/day (95%CI:0.97;4.43, p=0.002). By day 5 both groups had a mean blood pressure which was

significantly higher from their controls. Importantly in WT/WT mice, AngII induced an equivalent increase in blood pressure to that seen in control WT and Nox2^{-/-} mice [not shown]. The increase was 2.2mmHg/day (95%CI:0.52;3.84,p=0.01) which resulted in the blood pressure reaching a significantly higher value by day 5. This demonstrates transplantation per se does not affect the response of the mice to AngII infusion.

However different responses were seen in the WT/Nox2^{-/-} and Nox2^{-/-}/WT chimeric mice. In these chimeric mice, the increase in blood pressure to AngII stimulation was attenuated. The increase was 1.5mmHg/day and 1.6mmHg/day respectively. This meant that the blood pressure was not significantly higher until approximately day 10. However the increase in blood pressure was similar the two groups.

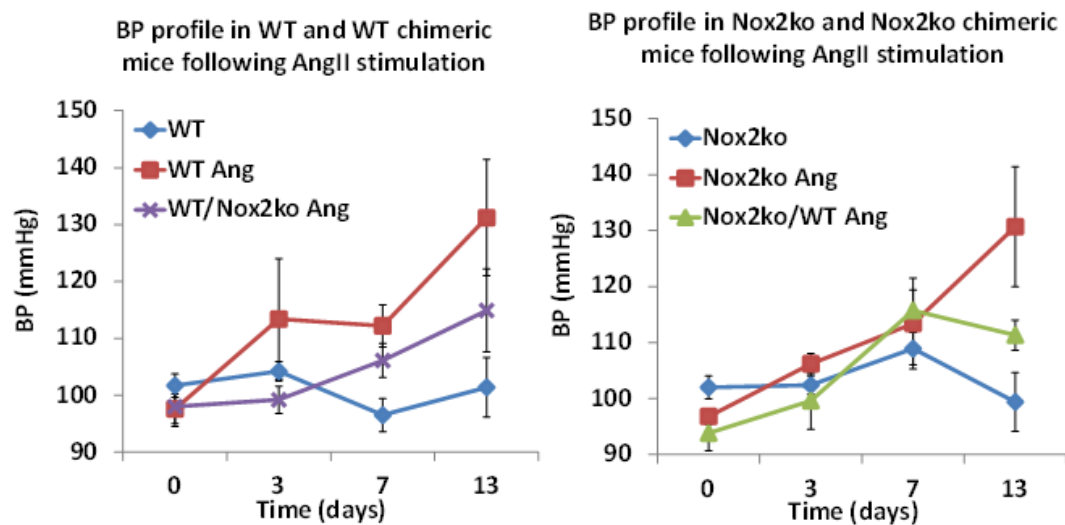


Figure 5-5 Blood pressure response following angiotensinII stimulation

AngII infusion resulted in a significant increase in systolic blood pressure in both WT (N=7) and Nox2^{-/-} (N=5) mice. However the increase in blood pressure in response to Ang II infusion in either WT chimeric mice with Nox2^{-/-} marrow (N=7) or Nox2^{-/-} chimeric mice with WT marrow (N=7) was attenuated.

5.3.3 *NOX2 IN CIRCULATING CELLS IS IMPORTANT FOR ANGII INDUCED CARDIAC HYPERTROPHY*

Following AngII stimulation both WT and Nox2^{-/-} hearts showed a significant increase in the left ventricular (LV)/body weight (BW) ratio compared to their respected unstimulated controls [1.00 (95%CI:0.3;1.7,p=0.01 and 1.02 (95%CI:0.28;1.76,p=0.01)]. Thus adjusted for their body weight the increase in the LV mass was 15.6mg (95%CI:1.05;34.2,p=0.045) and 17mg (95%CI:0.71;33.8,p=0.041) respectively. In line with the pressor response to AngII, WT/WT chimeric mice demonstrated a similar hypertrophic response to that in WT and Nox2^{-/-} mice (16.5mg (95%CI:1.01;31.9,p=0.037)). The LV/BW ratio increased by 1.00 (95%CI:0.27;1.73,p=0.01) thus confirming that the bone marrow transplantation procedure had not altered the ability to mount a hypertrophic response to AngII in these mice.

In marked contrast in WT/Nox2^{-/-} chimeric mice the hypertrophic response was attenuated (3.6mg (95%CI:-13.1;20.3,p=0.66)) and no longer showed a significant increase compared with WT control mice. The LV/BW ratio increased by 0.5 (95%CI:-0.25;1.25,p=0.16).

In contrast Nox2^{-/-}/WT chimeric mice demonstrated a substantial hypertrophic response (30mg (95%CI:12.2;47.9,p=0.002) compared to Nox2^{-/-} control mice. There was an increase in the LV/BW ratio of 2.1 (95%CI:1.35;2.85,p<0.001). This increase was larger (12.8mg (95%CI:-3.13;28.7,p=0.11)) than that seen in WT/WT chimeric mice. It is noteworthy that this increased hypertrophy was observed in mice which showed a reduced increase in their blood pressure.

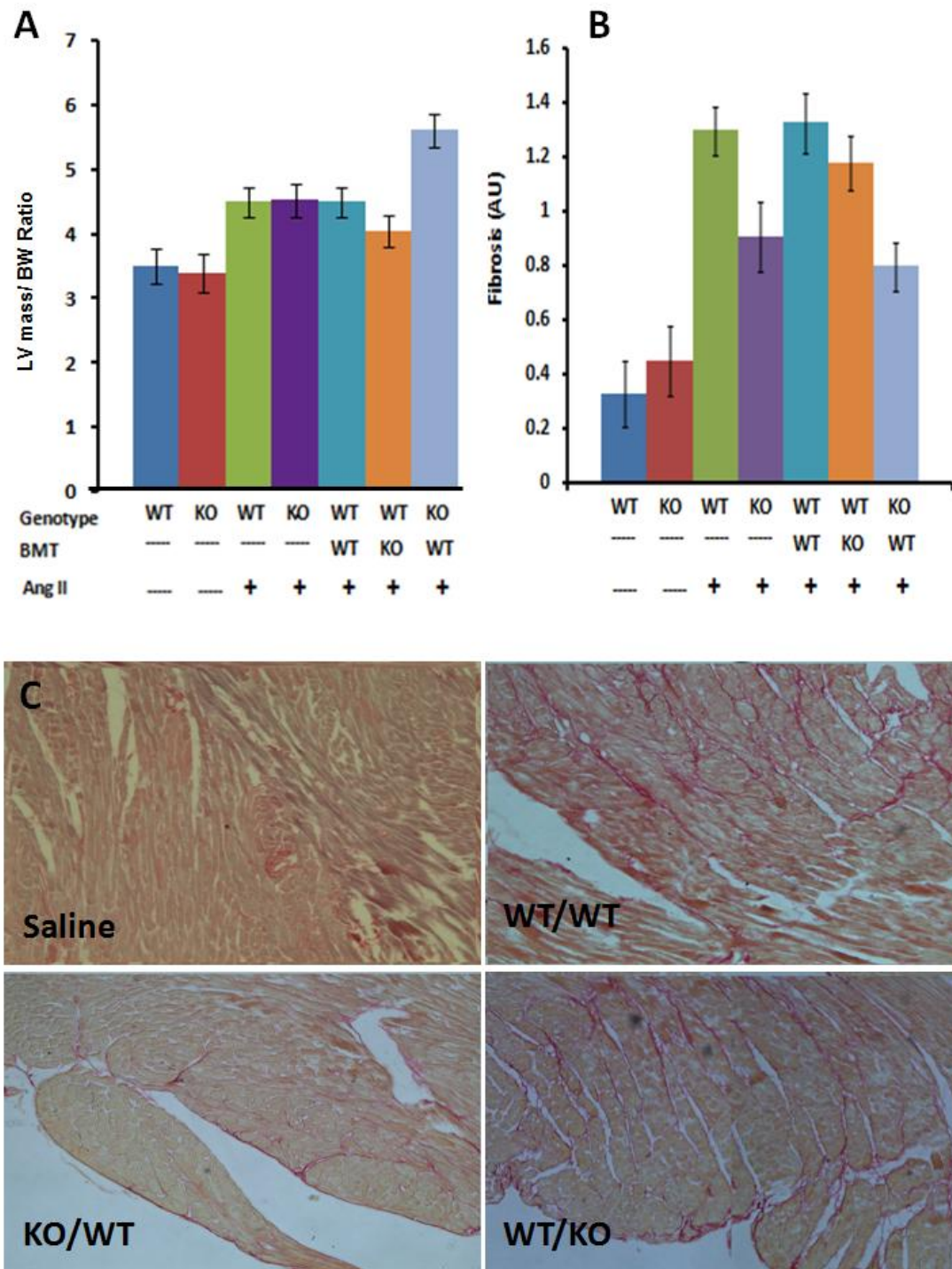


Figure 5-6 LV mass/Body weight ratio and fibrosis in chimeric mice following AngII stimulation

A: The LV/BW ratio increased significantly following AngII stimulation in WT ($p=0.01$) and Nox2^{-/-} ($p=0.01$) mice. No loss in response was observed following bone marrow transplantation as AngII stimulation resulted in a similar significant increase in the LV/BW ratio of WT/WT chimeric mice ($p=0.01$) compared to WT. However compared to WT, the selective loss of Nox2 in the circulating blood cells, namely in the WT/Nox2^{-/-} chimeric mice, prevented a significant increase ($p=0.16$) in LV/BW ratio

following AngII stimulation. Unexpectedly Nox2^{-/-}/WT demonstrated a significant ($p<0.001$) increase in its LV/BW ratio following AngII stimulation.

B & C: Cardiac fibrosis was visualized following staining of histology sections with Picosirus Red. The level of fibrosis was quantified and summarized in the top right graph. Following AngII stimulation significant ($p=0.002$) cardiac fibrosis was only observed in WT mice thus confirming earlier studies where Nox2 was found to be important in AngII induced cardiac fibrosis. Significant cardiac fibrosis was observed in WT/WT chimeric mice demonstrating once more that the process of generating chimeric mice had no effect on the physiological/pathophysiological response to AngII. Levels of cardiac fibrosis were unaltered in WT/Nox2^{-/-} ($p=0.39$) but remained significantly ($p=0.003$) reduced in Nox2^{-/-}/WT chimeric mice suggesting a crucial role for the Nox2 in the resident cardiac cells in AngII induced cardiac fibrosis. (N=6/7 mice were used in each group for experimentation)

5.3.4 NOX2 IN CARDIAC CELLS IS IMPORTANT FOR ANGII INDUCED CARDIAC FIBROSIS

AngII stimulation resulted in a significant increase in fibrosis in both the WT and WT/WT chimeric mice. The increase was approximately 0.82units (95%CI:0.34;1.3, $p=0.002$) greater than in vehicle infused WT mice. In line with earlier results no significant increase in fibrosis was seen following AngII infusion in Nox2^{-/-} mice (Increase in fibrosis of +0.41units (95%CI:-0.13;0.94, $p=0.127$) compared with vehicle infused Nox2^{-/-} mice). These results again support the hypothesis that AngII induced cardiac fibrosis is Nox2 dependent. In addition, they demonstrate once more that the process of generating chimeric mice had no effect on the physiological/pathophysiological response to AngII (Figure 5.6).

The selective loss of Nox2 in the circulating blood cells, namely in the WT/Nox2^{-/-} chimeric mice, had no significant effect on the degree of AngII induced cardiac fibrosis (figure 5.6). (WT/Nox2^{-/-} chimeric mice showed level of fibrosis -0.15 units (95%CI:-0.21;0.51, $p=0.39$) compared with WT/WT chimeric mice). In contrast, Nox2^{-/-}/WT chimeric mice maintained a

significantly reduced fibrosis compared with WT/WT (level of fibrosis was - 0.53 units (95%CI:0.21;0.86, $p=0.003$)) which was not significantly different from that seen in Nox2^{-/-} mice with or without AngII stimulation.

Taken together these results suggest that the Nox2 in the peripheral blood cells is not essential for the development of AngII induced cardiac fibrosis while Nox2 in the resident cardiac cells appears crucial in this process.

5.3.5 MONOCYTES WITH HIGH CD11B EXPRESSION (INFLAMMATORY PHENOTYPE) WERE OBTAINED FROM BLOOD AND BONE MARROW BY POSITIVE SELECTION

In mice the monocyte population in the blood comprises only 3% of the total leukocyte population and therefore the use of positive selection by microbeads was necessary in order to determine the characteristics of this cellular population. FACS for CD11b positive cells on the total cell population prepared from blood, i.e. before microbead selection, showed very few cells with a high expression of CD11b. (Figure 5.7 A) However following positive selection with the microbeads a large population of high CD11b expressing monocytes was obtained (Figure 5.7 B) and moreover FACS of the flow-through from the MACS Column showed very few CD11b positive expressing cells were lost. (Figure 5.7 C) Thus the process successfully captured the low numbers of high CD11b expressing cells with very few lost during washing providing a relatively pure population of monocytes.

There was relatively more high CD11b expressing cells in the bone marrow (Figure 5.7 D & F). Nevertheless positive selection with CD11b-microbeads on the bone marrow preparation resulted in a purer

concentration of high CD11b expressing monocytes. (Figure 5.7 E & F) As the bone marrow had a larger number of such cells, FACS for CD11b positive cells on the flow-through showed the loss of these cells. (Figure 5.7 F)

The possible contamination of the monocyte population selected by the microbeads with lymphocytes which express lower levels of CD11b was investigated. Double staining of CD11b and CD3 (pan leukocyte marker) was undertaken on the purified monocytes from blood and the flow-through from the MACS column. The blood sample showed no low CD11b and CD3 expressing cells. (Figure 5.7 G) However, these cells were observed in the flow through which suggest that they were not retained by the microbeads (possibly because the CD11b expression was too low) and were therefore subsequently lost in the washing (Figure 5.7 H-white arrow). In conclusion these experiments demonstrated that monocytes were successfully obtained and were thus used in future experiments.

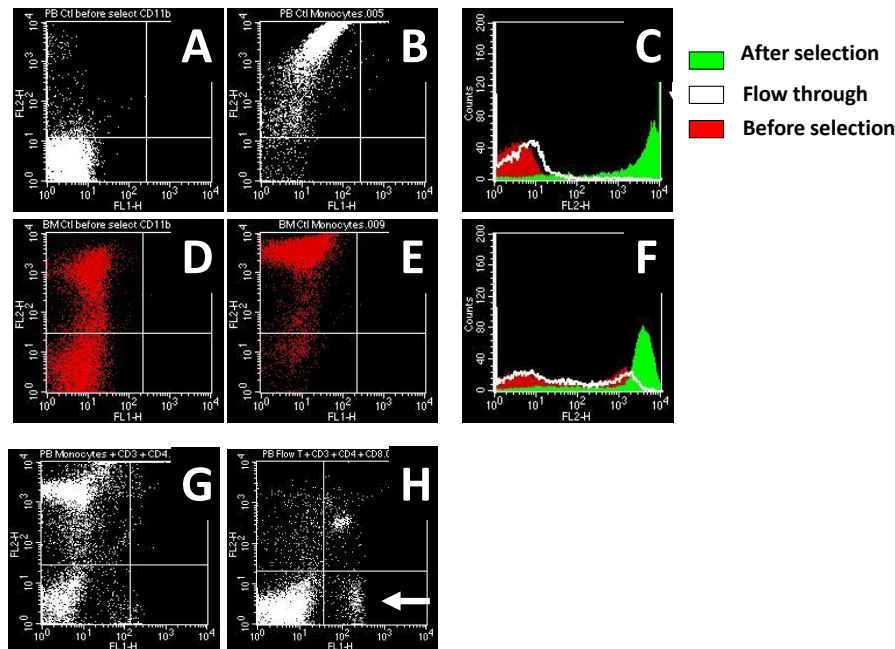


Figure 5-7 FACS analysis on blood and marrow monocytes before and after microbead separation

Blood:- **A:** Shows the scatter of blood cells before microbead separation. The y-axis shows the separation of mononuclear cells into those with high expression of CD11b (upper left quadrant). Few monocytes (CD11b high expressing) are observed. **B:** Following separation a concentration of monocytes is now observed in the left upper quadrant. **C:** The results of the separation are summarized. Few monocytes (CD11b high expressing) are observed before separation (red) however following separation CD11b expressing cells have been successfully concentrated (green). The loss of monocytes in the flow through of the MACS columns (white) shows minimal loss of these cells.

Bone marrow:- **D:** Bone marrow has a larger population of mononuclear cells with high CD11b expression even before MACS separation. **E:** However a homogenous population of monocytes (CD11b high expressing) cells is obtained following MACS separation as observed with a loss of cells in the lower left quadrant. **F:** The results are summarized where a large number of CD11b high and low expressing cells are observed amongst the mononuclear cells before separation (red). Following separation (green) a concentration of monocytes (CD11b high expressing) are obtained. Due to the large numbers of monocytes some were found in the wash through from the MACS columns.

Lymphocyte contamination:- **G:** Lymphocytes are the other common mononuclear cell population typically with low CD11b expression. As before the y-axis shows CD11b expression whereas now the x-axis is labeled to detect levels of CD3 expression (double staining). No discernible lymphocyte contamination (lower right quadrant) was found in the monocytes separated from blood. **H:** However the lymphocytes (CD3 positive

and low CD11b expressing) were found in the wash through from the MACS Column (white arrow).

5.3.6 ANGII STIMULATION RESULTED IN AN INCREASE IN L-SELECTIN EXPRESSION IN BLOOD MONOCYTES IN NOX2^{-/-} MICE

Modulation of L-selectin expression by AngII was investigated as discussed earlier, this selectin is important for the adherence of cells to the endothelium and their shedding and subsequent migration into tissue (Carlos and Harlan, 1994). Hence Nox2 modulation of its expression may influence the ability of cells to infiltrate the heart and induce cardiac fibrosis.

However, no significant differences in the baseline expression of L-selectin on bone marrow monocytes from WT (476AU +/- 21) and Nox2^{-/-} (484AU +/-13) mice could be detected. The expression of L-selectin was also similar between WT (547AU +/- 9) and Nox2^{-/-} (538AU +/- 19) monocytes derived from blood. (Figure 5.8) This would suggest that the baseline expression of L-selectin in blood and bone marrow derived monocytes was not significantly influenced by the loss of Nox2. Interestingly, independent of Nox2, the expression of L-selectin was found to be significantly increased in monocytes derived from blood as compared to bone marrow (average mean fluorescence was higher by 61.9AU (95%CI: 17.6; 106.2 p=0.012).

AngII stimulation did not induce any significant change in the expression of L-selectin in monocytes derived from blood or bone marrow in WT mice. Similarly in the Nox2^{-/-} mice the bone marrow derived monocytes did not show any significant change in the L-selectin expression with AngII stimulation. However L-selectin expression was significantly increased in blood monocytes derived from Nox2^{-/-} mice (by 83AU (95%CI: 37.8;

129.1, $p=0.001$). Therefore the data suggests a significant role of Nox2 in the shedding of L-selectin in peripheral blood monocytes following AngII stimulation. After 14 days of stimulation with AngII this manifested itself as an increase in the expression of L-selectin in Nox2^{-/-} peripheral blood monocytes.

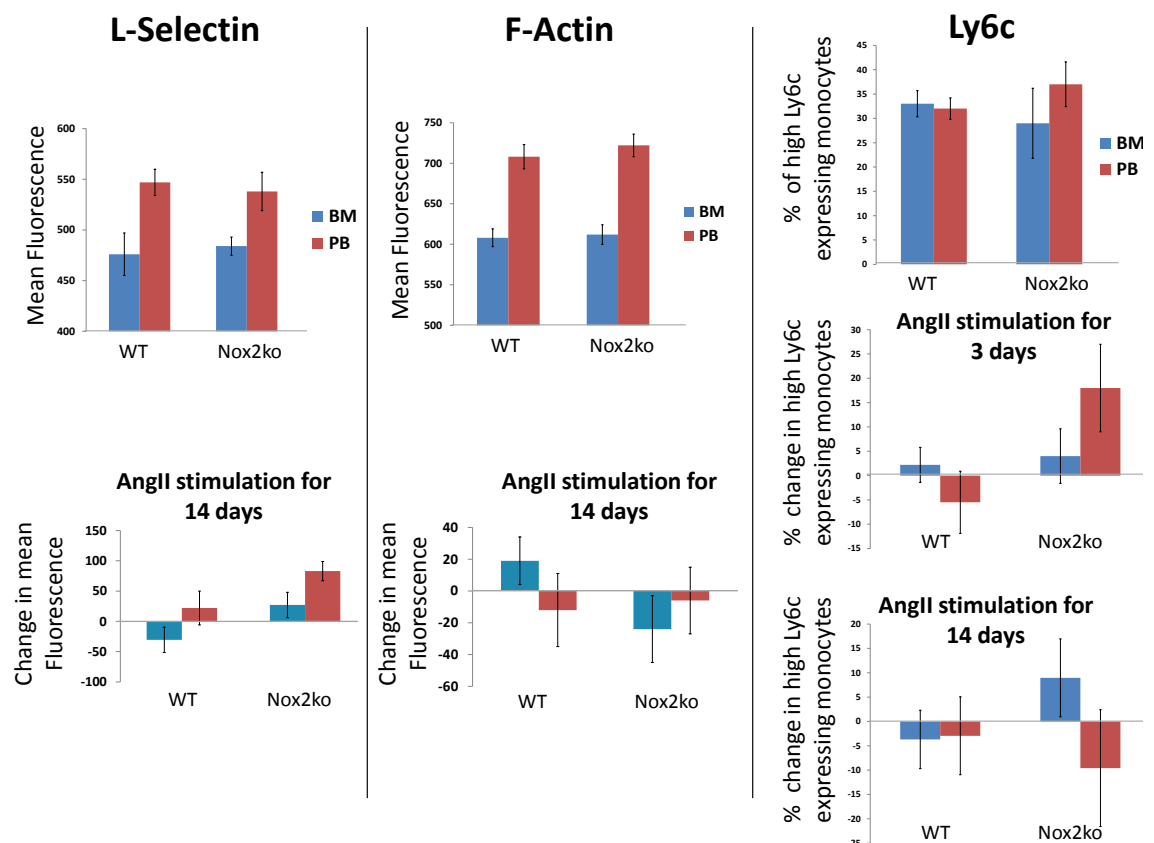


Figure 5-8 Changes in L-selectin, F-actin and Ly6c expression in WT and Nox2^{-/-} bone and blood monocytes following AngII stimulation

L-Selectin: Expression of L-selectin was significantly more in PB than BM in both WT and Nox2^{-/-} mice. Following AngII stimulation for 14 days expression of L-selectin was significantly increased in peripheral blood monocytes from Nox2^{-/-} mice.

F-Actin: Expression of filamentous actin was significantly higher in blood monocytes as compared to bone marrow. The loss of Nox2 or stimulation with AngII did not significantly change its expression.

Ly6c: Percentage of high Ly6c expressing monocytes was similar in the blood and bone marrow of WT and Nox2^{-/-} mice. Stimulation with AngII showed a significant increase in the percentage of high Ly6c expressing monocytes in Nox2^{-/-} by day 3. The percentages in both WT and Nox2^{-/-} mice were not significantly different from unstimulated by day 14.

5.3.7 F-ACTIN EXPRESSION WAS SIMILAR IN WT AND Nox2^{-/-} BLOOD AND BONE MARROW MONOCYTES FOLLOWING ANGII STIMULATION

The redox sensitive regulation of the actin cytoskeleton has been discussed in Chapter 3. Alterations in monocyte shape are important to enable the cell to migrate (Kamei and Carman, 2010; Muller, 2003) and in order to achieve this filamentous actin (F-actin) is changed to globular actin as the actin is broken down and recycled. Hence the expression levels of F-actin in WT and Nox2^{-/-} monocytes from blood and bone marrow were investigated.

In line with the L-selectin results, there was no significant difference in the baseline expression of F-actin in monocytes derived from bone in WT (608u +/- 11) or Nox2^{-/-} (612u +/- 15) mice. The expression of F-actin was also similar between monocytes derived from blood in WT (708 +/- 12) or Nox2^{-/-} (723 +/- 14). (Figure 5.8) As with L-selectin, this would suggest that the baseline expression of F-actin in blood and bone marrow derived monocytes was not significantly influenced by the loss of Nox2. Similar to L-selectin, independent of Nox2, the expression of F-actin was found to be significantly higher in monocytes derived from blood as compared to bone marrow. On average the mean fluorescence was higher by 105U (95%CI: 74;137 p<0.001).

However, in contrast to the L-selectin results, no significant change in the levels of F-actin in blood and bone marrow derived monocytes from either WT or Nox2^{-/-} mice was observed following stimulation with AngII for 2 weeks.

5.3.8 *LY6C EXPRESSION WAS INCREASED FOLLOWING 3 DAYS OF ANGII*

Monocytes with a high expression of Ly6c have been termed ‘inflammatory’ monocytes due to their preferential recruitment into tissue with ongoing inflammation (Nahrendorf et al., 2007; Panizzi et al., 2010). The recruitment of monocytes into the heart is an important first step in the development of cardiac fibrosis (Sun et al., 2002b). As Nox2 has been shown to be important in AngII induced cardiac fibrosis, the role of Nox2 in monocytes maturation, as observed by changes in Ly6c expression, in the bone marrow and blood could be important in the development of fibrosis. (Figure 5.8)

In WT mice the percentage (Figure 5.8 Ly6c-top) of high Ly6c expressing monocytes was similar in blood and bone marrow. The loss of Nox2 did not result in any significant change in the percentage of high Ly6c expressing monocytes in blood or bone marrow. Thus the global loss of Nox2 did not appear to influence the natural maturation process of monocytes in blood or bone marrow.

Following 3 days of AngII stimulation(Figure 5.8 Ly6c-middle) no significant change was observed in the percentage of high Ly6c expressing monocytes in WT blood or bone marrow. Similarly no significant change was observed in the percentage of high Ly6c expressing monocytes in the bone marrow of Nox2^{-/-} mice. However, in contrast, monocytes derived from blood in Nox2^{-/-} mice the percentage of high Ly6c expressing monocytes increased significantly by 18% (95%CI:-1.22;37.3,p=0.04).

Following 14 days of AngII stimulation the percentage of high Ly6c expressing monocytes in WT and Nox2^{-/-} blood and bone marrow showed no significant change as compared to mice which were not stimulated.

Thus any effect of the loss of Nox2 on the percentage of high Ly6c expressing monocytes following AngII stimulation could only be detected early during AngII stimulation and the differences were lost following more chronic stimulation.

5.4 DISCUSSION

5.4.1 *ROLE OF NOX2 IN CIRCULATING BLOOD CELLS AND RESIDENT CARDIAC CELLS IN CARDIAC HYPERTROPHY AND FIBROSIS*

MAIN FINDINGS:

- Nox2 in circulating peripheral blood cells is important in blood pressure control.
- Nox2 in circulating peripheral blood cells is crucial in AngII induced cardiac hypertrophy.
- Nox2 present in resident cardiac cells is crucial for AngII induced cardiac fibrosis.

Nox2^{-/-} mice developed less hypertrophy and fibrosis in response to AngII stimulation than wild type mice which confirmed previous results demonstrating an important role for Nox2 in this process. In this chapter the mechanism of this protective effect was further explored by investigating in which cell type was the loss of Nox2 critical. In order to do this, the already established globally deficient Nox2 mouse was used to create chimeric mice in which Nox2^{-/-} or WT circulating cells were combined with Nox2^{-/-} or WT mice.

AngII stimulation of WT chimeras with Nox2^{-/-} marrow and in Nox2^{-/-} chimeras with WT marrow resulted in a blunted blood pressure response. Nox2 is known to play an important part in AngII induced hypertension (Touyz, 2005) while other literature suggests that other Nox isoforms such as Nox1 are important (Gavazzi et al., 2006; Matsuno et al., 2005). As such Nox1 deficient mice display a blunted hypertensive response to AngII

(Gavazzi et al., 2006; Matsuno et al., 2005), whereas, Nox1 overexpression specifically in VSMCs potentiated AngII pressor response (Dikalova et al., 2005). In addition, cell types other than endothelium have been linked to hypertension, including vascular smooth muscle cells, adventitia and more recently T-cells (Dikalova et al., 2005; Guzik et al., 2007; Pagano et al., 1997). Furthermore, NADPH oxidase in systems other than the cardiovascular system, e.g. the CNS, appear also to play a part in hypertension (Zimmerman et al., 2004). The results in this chapter suggest that Nox2 in the circulating inflammatory cells has a role in the development of AngII induced hypertension as the absence of Nox2 in the WT/Nox2^{-/-} chimeric mice resulted in a blunted blood pressure response.

The blunted hypertensive response in Nox2^{-/-}/WT BM chimeric mice was unexpected. Byrne et al (Byrne et al., 2003) had described how Nox2^{-/-} mice have a natural lower baseline blood pressure necessitating the need to conduct experiments with pressor doses of AngII and thus again demonstrating an important role for Nox2 in the control of blood pressure. However, with pressor doses of AngII the global Nox2^{-/-} mice were able to demonstrate a hypertensive response similar to that observed in WT mice. The blunted blood pressure response in Nox2^{-/-}/WT chimeric mice, to pressor doses of AngII, would therefore suggest that the mechanism whereby AngII induces the hypertensive response in global Nox2^{-/-} mice could be different from that in WT mice. Normally NADPH oxidase is present in many different cells and tissue important for blood pressure control (Dikalova et al., 2005; Gavazzi et al., 2006; Guzik et al., 2007; Matsuno et al., 2005; Pagano et al., 1997; Touyz, 2005) and as we observed earlier in WT/Nox2^{-/-} chimeric mice that the presence of Nox2 in the peripheral circulating blood is

particularly important. Global Nox2^{-/-} mice have a different adapted physiology (Bendall et al., 2002b) especially with respect to the expression of other NADPH oxidases and antioxidants in cells and tissues which are important for the control of blood pressure. Thus the underlying mechanism by which a global Nox2^{-/-} mice would develop hypertension with pressor doses of AngII could be different from WT mice such that paradoxically the blood pressure response is attenuated by the presence of Nox2 in the peripheral circulating blood cells.

AngII induced cardiac hypertrophy was unaffected by the global loss of Nox2. Previous studies using global Nox2 knockout mice have shown that Nox2 is important in the development of cardiac hypertrophy in response to a low sub-pressor dose of AngII (Bendall et al., 2002a) and also at pressor doses (Byrne et al., 2003). However, the mice in these experiments (Bendall et al., 2002a; Byrne et al., 2003) had been stimulated for one week as compared to two weeks in the current experiments. With the longer duration of stimulation or higher doses of AngII the protective effect of Nox2 appears overwhelmed. As such other studies too have found the loss of Nox2 to have no effect on the development of cardiac hypertrophy induced by a higher pressor dose of AngII following 2 weeks stimulation (Grieve et al., 2006; Johar et al., 2006; Maytin et al., 2004). Taken together the results would suggest that the loss of Nox2 slows the development of AngII induced cardiac hypertrophy. Thus in global knockout mice the cardiac hypertrophy response is slower but with time a similar level of hypertrophy can be achieved. The key result of the current studies was the fact that selective loss of Nox2 from the circulating inflammatory cells resulted in an almost complete loss of cardiac hypertrophy despite 2 weeks of pressor dose of AngII stimulation.

This suggests that Nox2 in the circulating cells plays a critical role in inducing cardiac hypertrophy in response to AngII.

Transplantation of WT marrow into a Nox2^{-/-} mouse resulted in an increased AngII induced cardiac hypertrophy over and above that seen in WT mice. It is important to note that the global Nox2^{-/-} have a different adaptive physiology with respect to their redox expression (Bendall et al., 2002b). The redox state of a cell is important in modulating cell signaling. Evidence for this comes from studies on rat cardiomyocytes in which AngII induced hypertrophy was abolished by adenoviral mediated expression of Cu/ZnSOD (Hingtgen et al., 2006b) Thus in global Nox2^{-/-} mice, with time, a pressor dose of AngII can overwhelm the protective effect from the loss of Nox2. However in the Nox2^{-/-}/WT model we have the acute infusion of WT peripheral cells into Nox2^{-/-} mice with an altered redox state of their resident cells. The resident cardiac cells in the Nox2^{-/-} mice with their reduced antioxidant reserves (Bendall et al., 2002b) are now infiltrated by WT peripheral cells which we have observed to have a increased ROS response (compared to Nox2^{-/-} cells) following PMA (downstream signaling molecule for AngII) stimulation. Though the exact mechanism is not completely elucidated from these experiments, the net effect was an exacerbated hypertrophic response. Nevertheless, both these chimeric mice provide further support for a critical role of Nox2 in the circulating inflammatory cell in the development of AngII induced hypertrophy.

The blood pressure response in both WT/Nox2^{-/-} and the Nox2^{-/-}/WT chimeric mice was slower. The exaggerated hypertrophic response in Nox2^{-/-}/WT mice and the lack of it in WT/Nox2^{-/-} was in contrast. The result was in keeping with Bendall et al (Bendall et al., 2002a) who had shown a

reduced level of cardiac hypertrophy in the Nox2^{-/-} mice with sub-pressor doses of AngII suggesting that independent of the mechanical effect of blood pressure, chemical stimulation by angiotensin II induces cardiac hypertrophy via Nox2 oxidase. In contrast to AngII induced hypertrophy, the hypertrophic response to aortic banding was found to be similar between Nox2^{-/-} and wild type mice by Byrne et al (Byrne et al., 2003). They also found an increased level of NADPH oxidase activity which they ascribed to the increased levels of Nox4 seen in the Nox2^{-/-} mice. Maytin et al (Maytin et al., 2004) have also shown that pressor effects from aortic banding induced similar levels of cardiac hypertrophy in both wild type and Nox2^{-/-} mice. These authors also found increased levels of p22^{phox} and p47^{phox} mRNA. Taken together these data support the fact Nox2 does not play an essential role in cardiac hypertrophy as induced by the mechanical effect of aortic banding.

The selective loss of Nox2 from circulating inflammatory cells resulted in no change in the level of AngII induced cardiac fibrosis. This suggests that the inflammatory cells were not the critical cell source for the Nox2 in AngII induced cardiac fibrosis. However, the loss of Nox2 from the resident cardiac cells resulted in a loss in cardiac fibrosis demonstrating these cells as the critical cell source for AngII induced cardiac fibrosis.

This result supports the observation of a dissociation of the fibrotic response from that of the hypertrophic response to AngII. In models of infra-renal aortic banding with normal circulating levels of AngII and aldosterone, there is no evidence of increased cardiac fibrosis despite the presence of significant hypertrophy (Brilla et al., 1990). Similarly, chronic elevation of noradrenaline levels is not associated with increased cardiac fibrosis despite an elevation in blood pressure (Patel et al., 1989). Furthermore, in human

hypertension, fibrosis has been found in both the left and right ventricles in biopsies of myocardial tissue, despite the right heart pumping blood against a reduced afterload, suggesting that humoral factors are more important than the mechanical loading in the bringing about the fibrotic response (Amanuma et al., 1994). Raised levels of AngII are thought to play a key role in triggering the inflammatory response resulting in fibrosis. In support of this many studies have shown that the blockade of AngII or its action results in a reduction in fibrosis. For example, Tokuda et al (Tokuda et al., 2004) demonstrated that a sub-depressor dose of candesartan (an angiotensin II receptor blocker) in mice who had had supra-renal aortic banding markedly ameliorated perivascular fibrosis. The differential role of Nox2 in peripheral cells in promoting cardiac hypertrophy and in resident cardiac cells in promoting cardiac fibrosis following AngII stimulation offers a mechanism by which cardiac hypertrophy and fibrosis could occur independently. This observation therefore has the potential as a specific therapeutic target for the management of cardiac hypertrophy and fibrosis separately. In hypertensive heart disease the unwanted fibrotic effects could potentially be targeted specifically.

The critical cellular source for Nox2 in AngII induced cardiac fibrosis could be one or all of three possible sources. Firstly the resident fibroblasts were investigated and discussed in chapter 2 by way of their NADPH oxidase dependent ROS response. A Nox2 dependent ROS response was not observed following AngII stimulation. However, given that a Nox2 dependent ROS response observed in cardiac fibroblasts following TGF β stimulation and the downstream placement of TGF β to the effects of AngII (Schultz et al., 2002), could establish cardiac fibroblasts as an important cellular source for Nox2.

Secondly cardiac myocytes are known to express Nox2 (Bendall et al., 2002a; Peng et al., 2005; Xiao et al., 2002) and in cultured cells and *in vivo* models the activation of cardiomyocyte Nox2 may contribute to the development of AngII induced cardiac hypertrophy (Bendall et al., 2002a). A similar critical role for cardiomyocytes Nox2 could exist in cardiac fibrosis thus making it a critical cellular source for Nox2. Finally, the endothelium could be an important cellular source for Nox2 in the development of cardiac fibrosis. The endothelium in pathologic settings, such as in the renin-angiotensin system activation, can become activated and promote increased interaction with circulating inflammatory cells (Frey et al., 2009). The infiltration of inflammatory cells is an important early event in the development of cardiac fibrosis, making this a possible important cellular source of Nox2. Another mechanism by which endothelial Nox2 might promote cardiac fibrosis is through the process of endothelium mesenchymal transition (EndoMT). EndoMT has been recognized as an important source of cardiac fibroblasts and was discussed in chapter 2. Zeisberg et al (Zeisberg et al., 2007a) showed in a landmark study that EndoMT makes a significant contribution to pathologic cardiac fibrosis in a mouse model of transverse aortic constriction. Whether endothelial Nox2 activation affects EndMT and is therefore a critical cellular source of Nox2 in AngII induced cardiac fibrosis needs to be investigated.

5.4.2 *CHANGES IN F-ACTIN & L-SELECTIN EXPRESSION AND IN THE NUMBERS OF LY6C^{HI} EXPRESSING MONOCYTES*

MAIN FINDINGS:

- Bone marrow derived monocytes express less L-Selectin and F-Actin but have a similar number of Ly6c expressing cells in comparison to blood derived monocytes.
- Nox2 is not important in the baseline expression of L-Selectin, F-Actin or Ly6C.

As the infiltration of monocytes/macrophages into the heart is an important initial step in the development of cardiac fibrosis (Kuwahara et al., 2003) the characteristics of these cells from WT and Nox2^{-/-} mice were investigated. High Ly6c expressing ‘inflammatory’ monocytes preferentially migrate into sites of inflammation (Geissmann et al., 2003; Qu et al., 2004). L-selectins are important for the homing of cells to sites of inflammation (Kishimoto et al., 1989) where the monocytes can adhere and change shape enabling them to migrate into tissue, a process that requires the shedding of L-selectin. Thus the numbers of high Ly6c expressing monocytes and the expression of L-selectin and F-actin in blood and bone marrow monocytes was investigated following AngII infusion.

5.4.2.1 Actin

Monocytes in the bone marrow are adherent to the marrow sinusoid. On migrating out of the marrow their shape is spherical as they are suspended in the blood. In both WT and Nox2^{-/-} mice F-actin expressions were higher in monocytes from blood as compared to bone marrow. The increase in F-actin may reflect the necessary change in the cytoskeleton as the monocyte changes shape from being flat to spherical. The flat monocyte has to migrate and hence requires relatively more of its actin to be in the globular, rather than in filamentous form, to enable for actin cytoskeleton turn over at the leading edge. The actin cytoskeleton and its regulation, along with the important role of ROS in its modulation, has been described in Chapter 2. Here, we observe that the lack of any baseline difference in F-actin levels between WT and Nox2^{-/-} monocytes from blood and marrow suggests a lack of importance of Nox2 derived ROS in regulating the baseline levels of F-actin.

Stimulation by AngII has been shown to lead to changes in the actin cytoskeleton. Godin et al (Godin and Ferguson, 2010) on stimulation of HEK293 cells with AngII were able to induce membrane blebbing. Again in HEK293 cells Cotton et al (Cotton et al., 2007) were able to demonstrate membrane ruffling and Barnes et al (Barnes et al., 2005) showed the formation of stress fibres. However, stimulation with AngII in our experiments did not show any significant change in the F-actin levels. The stimulation with AngII in our experiments had been for 2 weeks unlike the more acute stimulation used in the above papers. Our results demonstrate that though there may be acute effects of AngII stimulation on the

cytoskeleton, no significant quantitative change in F-actin levels are observed following chronic exposure as in hypertensive heart disease.

5.4.2.2 L-selectin

The higher expression of L-selectin in the blood monocytes as compared to bone marrow monocytes could reflect the necessity of the rolling and adhering process of these cells to the vascular endothelium. Evidence in the literature suggests that the adherence of neutrophils to endothelium increases with exposure to H₂O₂ (Fraticelli et al., 1996). Also metalloproteinase 17, a sheddase of L-Selectin, has been shown to be redox sensitive (Wang et al., 2009b). The regulation of the expression of L-selectin is redox sensitive. However, the loss of ROS and in particular that from Nox2 did not influence the baseline expression of L-selectin suggesting that a lack of Nox2 derived ROS not to be crucial in this process.

The role of AngII in the regulation of L-selectin shedding has been demonstrated from different lines of evidence. Clinical studies have shown that L-selectin density on lymphocytes and monocytes/macrophages were significantly lower in hypertensive patients than in normotensives (Mills et al., 2002; Zapolska-Downar et al., 2006). In an *in vitro* study Vega et al demonstrated that AngII, in a dose dependent manner, induced shedding of L-selectin in human neutrophils rapidly over the course of minutes reaching maximum shedding at five minutes (Vega et al., 2010). Thus, an AngII dependent mechanism might lead to direct L-selectin shedding and leukocyte extravasation into local inflammatory sites in hypertensive cardiac disease. However, stimulation with AngII did not result in any significant change in the L-selectin levels of WT blood monocytes.

Unexpectedly a significantly increased expression of L-selectin was observed in Nox2^{-/-} blood monocytes following AngII stimulation. The exact mechanism of this increase is difficult to explain but could involve less shedding. However, the effect of such an increase would be to impede the migration of these cells into the heart thus explaining the slower hypertrophic response observed in Nox2^{-/-} mice. This would require corroborating by undertaking histological staining of the hearts.

5.4.2.3 Ly6c

The bone marrow has its own environmental niche and committed progenitors tend to localize to their bone marrow regions (Lo et al., 2007; Lo et al., 2009; Lo and Scadden, 2011). Oxidative stress and redox dysregulation in the bone marrow have direct consequences in signalling cascades involving pathways of survival, proliferation, angiogenesis and metastasis (Giles, 2006; Gius and Spitz, 2006; Szatrowski and Nathan, 1991a). As such studies in the 1950s indicated a critical role for cysteines and thiols in bone marrow cell proliferation (Baldini and Sacchetti, 1953). Long-term, self-renewing haematopoietic stem cells (HSC) have low levels of intracellular ROS. However, high ROS may result in senescence, apoptosis and a failure to self renew. Mice deficient in ROS regulating genes have HSCs that cannot maintain quiescence or self-renewal capacities (Naka et al., 2007). The specific role of the ROS from Nox2 in the regulation of numbers of high Ly6c expressing monocytes was investigated.

The baseline percentage of high Ly6c expressing monocytes was not different between WT and Nox2^{-/-} mice suggesting that the global loss of Nox2 did not affect the proportion of inflammatory monocytes found in

blood or bone marrow. However, other data from our laboratory has shown that selective overexpression of Nox2 in endothelium resulted in a significantly higher percent (10%) of high Ly6c expressing monocytes in the blood with a corresponding significant lower percent (20%) in the bone marrow. (unpublished) This therefore suggests that Nox2 in the endothelium of the bone marrow may be important in regulating the release of these cells. The regulation of marrow cell trafficking has been reviewed by Shi (Shi, 2012) where the high Ly6c expressing monocytes are said to be released from the bone marrow in response to chemokine CCL2. The local production of CCL2 in the bone marrow can be induced by circulating T-Lymphocyte receptor ligands. Using CCL2 reporter mice, CCL2-expressing cells are identified at the perivascular sites and seem to attract monocytes to the abluminal aspect of the endothelium where they subsequently gain access to the circulation. The results discussed above show that Nox2 in the endothelium results in more inflammatory monocytes circulating in the blood with resultant increase in cardiac fibrosis, in contrast to WT mice.

Ly6C^{hi} monocytes are generated in the bone marrow, and can be rapidly recruited to the peripheral tissues during infection or inflammatory response. Disease processes such as MI and atherosclerosis have been shown to be associated with changes in high Ly6c expressing monocytes. Exposure to AngII underlies such changes supporting the possibility of a role for inflammatory monocytes in the development of hypertensive heart disease. However, no significant change was observed in the proportions of inflammatory monocytes following 3 days or 14 days of AngII stimulation in blood or marrow in WT mice. In Nox2^{-/-} mice the numbers of high Ly6c expressing monocytes did significantly increase after 3 days of AngII

stimulation in the blood, an effect which was lost at day 14. The marrow did not show any significant change. This unexpected finding is in keeping with the increased expression of L-selectin found in Nox2^{-/-} mice following AngII stimulation and could reflect an impaired tissue infiltration of these cells, the effect of which is overwhelmed by day 14. This would again be in keeping with the slower hypertrophic response in Nox2^{-/-} mice to AngII stimulation. Further investigation of the numbers of high Ly6c expressing monocytes in the blood at earlier time points and double staining and FACS analysis with L-selectin on high Ly6c expressing monocytes would help clarify this finding further.

In conclusion these experiments demonstrate that the Nox2 in the peripheral blood cells has a significant role in the development of cardiac hypertrophy, while that in the resident cells is crucial for the development of cardiac fibrosis, following AngII stimulation. The data also suggests a possible hypothesis explaining the delayed hypertrophic response observed in Nox2^{-/-} mice hearts following AngII stimulation

6 GENERAL DISCUSSION

6.1 BACKGROUND

Hypertensive heart disease results in the development of cardiac hypertrophy and fibrosis. In mouse models of hypertensive heart disease, simulated with AngII infusion, Nox2 has been shown by our laboratory to be important in this pathophysiological process (Bendall et al., 2002a; Byrne et al., 2003; Johar et al., 2006).

The development of cardiac fibrosis is thought to be in part the result of raised levels of AngII which can trigger an inflammatory response resulting in fibrosis. The fibrosis has been found in both the left and right ventricles in biopsies of myocardial tissue, despite the right heart pumping blood against a reduced afterload and not developing cardiac hypertrophy (Amanuma et al., 1994). This suggests that a humoral cause, namely AngII, for the development of cardiac fibrosis in the right ventricle. Also many studies have shown that the blockade of AngII or its action results in a reduction in fibrosis. As such Tokuda et al (Tokuda et al., 2004) demonstrated that a sub-pressor dose of candesartan (an AngII receptor blocker), in mice who had had supra-renal aortic banding, markedly ameliorated perivascular fibrosis.

Our laboratory has shown that Nox2^{-/-} mice had a markedly reduced fibrosis compared to wild type animals in response to either a subpressor or pressor infusion of AngII by Bendal et al, Byrne et al and Johar et al (Bendall et al., 2002a; Byrne et al., 2003; Johar et al., 2006). Furthermore, Johar et al (Johar et al., 2006) showed that the profibrotic effects of AngII were inhibited by the antioxidant N-acetyl-cysteine, consistent with the role of

ROS in this process. Nox2 has also been implicated in aldosterone induced myocardial fibrosis (Johar et al., 2006). Furthermore, Nox2 NADPH oxidase has been implicated in the genesis of fibrosis in several other organ systems (An et al., 2007; Bataller et al., 2003; Manoury et al., 2005; Rey and Pagano, 2002).

However, the role of Nox2 in cardiac fibrosis secondary to the pressor effect of aortic banding is conflicting. Matyn et al (Maytin et al., 2004) published evidence that Nox2 oxidase did not play an important role in cardiac fibrosis in this model as myocardial fibrosis was increased to a similar level in both wild type and Nox2^{-/-} mice. In contrast, Grieve et al (Grieve et al., 2006) demonstrated a reduced level of fibrosis in the Nox2^{-/-} mice implicating an important role for the Nox oxidase in myocardial fibrosis following aortic banding.

Previous studies using global Nox2 knockout mice have shown Nox2 to be important in the development of cardiac hypertrophy in response to a low sub-pressor dose of AngII (Bendall et al., 2002a) and also at pressor doses (Byrne et al., 2003). However the mice in these experiments had been stimulated for one week as compared to two weeks in the current experiments. With the longer duration of stimulation or higher doses of AngII the protective effect of Nox2 appears overwhelmed. As such other studies too have found the loss of Nox2 to have no effect on the development of cardiac hypertrophy induced by a higher pressor dose of AngII following 2 weeks stimulation (Grieve et al., 2006; Johar et al., 2006; Maytin et al., 2004). Taken together, the results would suggest that the loss of Nox2 slows the development of AngII induced cardiac hypertrophy. In global knockout

mice the cardiac hypertrophy response is slower but with time a similar level of hypertrophy can be achieved.

In contrast to AngII induced hypertrophy, the hypertrophic response to aortic banding was found to be similar between Nox2^{-/-} and wild type mice by Byrne et al (Byrne et al., 2003). They also found an increased level of NADPH oxidase activity which they ascribed to the increased levels of Nox4 seen in Nox2^{-/-} mice. Maytin et al (Maytin et al., 2004) have also shown that pressor effects from aortic banding induced similar levels of cardiac hypertrophy in both wild type and Nox2^{-/-} mice. Taken together these data support the fact Nox2 does not play an important role in cardiac hypertrophy as induced by the mechanical effect of aortic banding, however, it does have a role in cardiac hypertrophy from the humoral effects of AngII. This alludes to different mechanisms in the development of cardiac hypertrophy from different stimulants. A key question was how Nox2 drives both AngII induced cardiac hypertrophy and fibrosis and in particular the critical cell source of the Nox2.

6.2 NOVEL FINDINGS FOR THE CELL SPECIFIC ROLE OF NOX2

Results from chapter 5 showed that the Nox2 in the circulating cells were important in the development of AngII induced cardiac hypertrophy and that the Nox2 in the resident cardiac cells were important in the development of cardiac fibrosis. This was a departure from the conventional understanding in hypertensive heart disease that cardiac fibrosis and hypertrophy are inevitably linked and developed together. The observation allowed for a possible underlying mechanism whereby the modulation of downstream signalling by ROS from Nox2 in different cell types could

orchestrate the development of the two processes separately. Thus, following AngII stimulation the selective lack of redox modulation, by way of ROS from Nox2, in the circulating cells could lead to the development of cardiac fibrosis without hypertrophy. Conversely following AngII stimulation the lack of redox modulation, by way of ROS from Nox2, in the resident cardiac cells could lead to the development of cardiac hypertrophy without cardiac fibrosis.

The independent development of cardiac fibrosis and hypertrophy is supported in the literature. Thus in models of infra-renal aortic banding with normal circulating levels of AngII and aldosterone, there is no evidence of increased cardiac fibrosis despite the presence of significant hypertrophy (Brilla et al., 1990). Similarly, chronic elevation of noradrenaline levels, resulting in an elevation in blood pressure and cardiac hypertrophy, is not associated with increased cardiac fibrosis (Patel et al., 1989). Furthermore as stated earlier in human hypertension fibrosis has been found in both the left and right ventricles despite the right heart pumping blood against a reduced afterload and not developing cardiac hypertrophy (Amanuma et al., 1994).

The cell-specific role of the Nox2 was an interesting aspect of the current study. This has clinical relevance as some aspects of the hypertrophic response are considered protective or adaptive in the face of increased wall stress and therefore beneficial at least for a period of time (Frey and Olson, 2003). However, the development of cardiac fibrosis and subsequent diastolic dysfunction is detrimental in hypertensive heart disease and the ability to target an individual component of the disease may be valuable.

The cardiac fibroblasts produce collagen during development of cardiac fibrosis and hence were a natural choice as an important cell source

for Nox2 in the development of AngII induced cardiac fibrosis. The cardiac fibroblasts were investigated for their ROS response to stimulation by AngII, aldosterone and TGF β , in chapter 2, all stimulants having been implicated in the development of fibrosis. A Nox2 dependent ROS response in the cardiac fibroblasts to these stimulants would suggest behaviour in vitro which in vivo would implicate the resident adult cardiac fibroblasts as a critical cell source for Nox2. However, no Nox2 dependent ROS response was elicited by AngII or aldosterone stimulation. This suggested that, in hypertensive heart disease following AngII stimulation, Nox2 in the resident cardiac fibroblasts may not be a critical cell source.

A recent study suggested a different mechanism whereby endothelial cells contribute, at least in part, to fibrosis by demonstrating endothelial to mesenchymal transition (EndMT) (Zeisberg et al., 2007a). Adult fibroblasts are thought to be derived directly from embryonic mesenchymal cells (Maric et al., 1997). Zeisburg et al (Zeisberg et al., 2007a) showed that appearance of fibroblasts during cardiac fibrosis was not solely a result of proliferation of resident fibroblasts but that there was a significant component which had an endothelial origin. The paper suggests that the endothelial cells, and in particular those that undergo EndMT to fibroblasts, are important in the development of cardiac fibrosis and thus could be the crucial resident cell source for Nox2.

It is known that adult cardiac fibroblasts contribute to cardiac fibrosis by transforming to myofibroblasts and increasing the deposition of ECM (Sun et al., 2002a). TGF β_1 signalling via Smad2/3 is important for this transformation and Nox4 has been shown to be important in this signalling process (Cucoranu et al., 2005). On stimulation of human cardiac fibroblasts

with TGF β_1 , Nox4 mRNA expression was significantly increased and ROS production significantly decreased by siRNA against Nox4. Moreover in a hallmark paper, Schultz et al (Schultz et al., 2002) tested whether AngII would be able to induce cardiac hypertrophy and fibrosis in vivo in the absence of TGF β_1 gene. The lack of the TGF β_1 gene fully prevented the development of cardiac hypertrophy and dysfunction induced by subpressor doses of AngII that was observed in wild type mice. This study provided the first direct evidence that AngII induced cardiac hypertrophy is mediated by TGF β_1 and indicate that TGF β_1 acts downstream of AngII and promotes myocyte growth and fibrosis in the heart.

The murine adult cardiac fibroblasts were stimulated with TGF β_1 in chapter 2 and demonstrated a Nox2 dependent ROS response. This finding placed Nox2 downstream of TGF β_1 and indicated that the fibroblasts could still be an important cell source of the Nox2 in the development of cardiac fibrosis in mice following AngII stimulation. Further in vivo and in vitro studies would be necessary in establish the exact role of TGF β_1 signalling via Nox2 in the development of cardiac fibrosis.

Another key result of the current studies was the fact that selective loss of Nox2 from the circulating inflammatory cells resulted in a loss of cardiac hypertrophy despite 2 weeks of pressor dose of AngII stimulation. This suggests that Nox2 in the circulating cells plays a critical role in inducing cardiac hypertrophy in response to AngII. Infiltration of the heart by macrophage/monocytes, in the circulating blood, is important for the development of cardiac fibrosis and hypertrophy (Kuwahara et al., 2003; Kuwahara et al., 2004). The role of Nox2 has been demonstrated to be important in the migration of endothelial cells and VSMC. The current study

demonstrated Nox2 to be crucial in the migration of macrophages in chapter 3 and thus indicated how the Nox2 in the peripheral circulating cells could be critical in the development of hypertensive heart disease by way of its role in macrophage migration. A key finding was the loss of chemotaxis in BMM towards CSF-1. The lack of chemotaxis in Nox2^{-/-} macrophages observed in-vitro could reflect in-vivo behaviour and thus explain the reduced hypertrophy and fibrosis which was observed. However, the migration of macrophages to chemoattractants in vivo is an integrated response to many other chemoattractants such as MCP1 and PDGF amongst others. The downstream signalling for all of them may not be Nox2 dependant thus offering a mechanism for the slower development of cardiac hypertrophy in Nox2^{-/-} mice observed in other studies. Further migration assays towards other chemoattractants would be necessary to further investigate the importance of Nox2 in macrophage migration.

Dunn chamber analysis of macrophage migration in chapter 3 allowed for the speed and persistence of the migration to be assessed along with chemotaxis. The novel observation was that, along with chemotaxis, both the speed and persistence of the macrophages is dependent on Nox2, as both were significantly reduced. Cellular migration is a complex procedure and many of the steps downstream from receptor activation by the chemoattractant are redox sensitive. Intracellular signal amplification to establish a gradient and direction by phosphoinositides, cdc42 in cellular polarisation and lamelopodia formation, cytoskeletal actin turnover, actin-myosin contractility and integrin adhesion have been shown to be redox sensitive and discussed in chapter 8. ROS from Nox2 by way of localised ROS production can theoretically modulate the migratory process at any of these

steps resulting in the reduction in cellular speed and persistence and the loss of chemotaxis. A more detailed investigation into the molecular mechanism whereby Nox2 modulates this process is a logical further direction for this research.

6.3 THERAPY

There are drugs currently in clinical use that are thought to exert some of their benefits by reducing oxidative stress by way of inhibition of Nox2 oxidase. Antagonism of the RAS is regularly undertaken when managing hypertension and hypertensive heart disease. ACE inhibitors and AngII receptor blockers (ARBs) are first line medication used in the control of hypertension. ACE inhibitors are used to reduce AngII levels in the treatment of cardiovascular diseases. However, as previously described, AngII is well known to be able to activate NADPH oxidase (Griendling et al., 1999). Clinical studies have demonstrated that the blockade of the RAS via treatment with ARB or ACE inhibitors reduces oxidative stress and inflammation (Dandona et al., 2006). In clinical studies, the selective inhibition of angiotensin II by ARBs can prevent end-organ damage from hypertension-associated diseases such as CHD, atherosclerosis, and renal disease, and these effects appear to be potentially independent of their BP-lowering effects. Thus reduction in endpoints were seen in patients with Non-insulin dependent diabetes mellitus with the Angiotensin II Antagonist Losartan [RENAAL], (Brenner et al., 2001) Irbesartan in Diabetic Nephropathy Trial [IDNT], (Lewis et al., 2001) Losartan Intervention For Endpoint reduction in hypertension [LIFE] study, (Dahlof et al., 2002) Candesartan cilexetil in Heart failure Assessment of Reduction Mortality and

morbidity [CHARM] study, (Pfeffer et al., 2003b) and VALsartan In Acute myocardial iNfarction Trial [VALIANT] (Pfeffer et al., 2003a). This further benefit of ARBs and ACEs may therefore be, at least in part, due to reduction in NADPH oxidase activation.

Statins have also shown to have antioxidative effects. Although the beneficial effects of statins have been mainly attributed to their cholesterol-lowering properties, there is growing evidence that some of the advantageous effects of these agents are independent of their plasma cholesterol lowering effect (Shepherd et al., 1995; Werner et al., 2002). One such mechanism is the inhibition of Rac1 (Cordle et al., 2005) which is pivotal for Nox2 activation. Therefore, statins may potentially exert some of their beneficial effects via inhibition of NADPH oxidase secondary to Rac1 inhibition (Maack et al., 1999).

Nevertheless, there remains a substantial morbidity and mortality even in patients treated with statins and antagonists of the RAS system. Recent evidence points to the interplay between hypercholesterolemia and hypertension, acting through the RAS, to increase cardiovascular risk (Nickenig and Harrison, 2002a; Nickenig and Harrison, 2002b; Sander and Giles, 2002). Oxidative stress has been implicated in many pathophysiological conditions in the cardiovascular system, including hypercholesterolemia, hypertension, diabetes, and heart failure (Lavy et al., 1991). There still remains room for additional approaches such as the targeting of NADPH oxidase in the management of such patients.

The current results raise the intriguing possibility that cell specific Nox2 and/or the pathways linked to its activation could provide a therapeutic target to tackle separately cardiac fibrosis and diastolic dysfunction and/or

hypertrophy especially regression of LVH. A clear caveat to this idea is being able to achieve inhibitor selectivity at the cellular and isoform level. Such selective inhibition should **not result in any unforeseen detrimental effects**.

6.4 STUDY LIMITATIONS

The studies in this thesis were performed in mice and any extrapolation to the clinical setting should clearly be made with caution in view of species differences. In humans, hypertension, and the resultant hypertensive heart disease, are generally diseases of middle age or beyond. The model that was chosen for study was AngII infusion for 2 weeks, which is an established experimental model associated with the development of significant interstitial cardiac fibrosis. Therefore, the characteristics of the process may be different even allowing for the difference in species.

The Nox2^{-/-} mice have a different adaptive physiology with respect to their redox expression (Bendall et al., 2002b). In keeping with this Byrne et al (Byrne et al., 2003) showed the increased levels of Nox4 expression in the heart of Nox2^{-/-} mice. The altered physiology would make it difficult to ascribe all of the effects in the results to the absence of Nox2. The underlying mechanism could for some of the results be due to, for example, the raised levels of Nox4 or other as yet unidentified protein. This point has been discussed in the appropriate chapter discussion.

In setting up the chimeric mice, the mice were irradiated to eradicate their marrow cells before replacement by tail vein injection. Again the use of chimeric mice is a well established method enabling the investigation of the effect a particular cell/gene of interest. However, the process of irradiation

could by itself induce biological responses which could have an influence in the development of hypertensive heart disease and is rarely observed in the past medical history of patients with hypertensive heart disease. The experiments were therefore undertaken with appropriate controls nevertheless the extrapolation of cell specific findings needs to be made with caution.

It is important to appreciate that the findings from the chimera experiment are currently only interpreted as those due to peripheral or resident cell. The marrow infusion by tail vein injection would invariably have a mix of other cells such as stem cells and endothelial progenitor cells. Clearly the studies do not identify the specific cell but only allow understanding of the effect of Nox2 in cells either resident or for those in the peripheral blood.

The investigation of the ROS response in cardiac fibroblast and the migration of BMM macrophages was undertaken in-vitro. Such a reductionist approach is clearly beneficial in allowing investigation of the response of interest to the single stimulation of interest. However, this environment is far removed from the in vivo experience where the cells are in tissue and exposed to a variety of stimulations at the same time. Whether the isolated responses observed also hold true in vivo depends on the outcome from the integration of all the stimulatory signals. The possibility exists that the responses observed in vitro may be, in in vivo, exaggerated due to a synergistic interaction or not significant due to redundancy in the signalling pathways.

6.5 FUTURE WORK

- A drawback of knockout mice is that the absence of the target gene can lead to compensation by other genes. Therefore, a logical study to confirm the findings in this thesis would be to make use of inducible knockout technology which could prevent any long-term compensatory effects induced by the absence of the gene, while still being able to achieve removal of the target gene for a specific time.
- There were some unexpected results from the current experiments. In particular the cellular work undertaken in chapter 2, 3 and 4 would require confirmation with siRNA inhibition of Nox2. This would not only confirm results but will also be a setup in which the Nox2 is inhibited acutely and will allow the effects to be ascribed more confidently to Nox2 rather than other compensatory mechanisms which may be the result of chronic loss of Nox2.
- The other option would be to generate cell specific Nox2^{-/-} or overexpressing mice to assess the effects of Nox2 in specific cells in AngII induced LVH and/or cardiac fibrosis. The specific knockdown or overexpression of Nox2 in circulating monocytes or in cardiac fibroblasts may be able to assess the extent to which endogenous Nox2 contributes to cardiac fibrosis and/or hypertrophy. This could of particular help in

investigating the unexpected finding in the blood pressure control in the chimeric mice.

- Another complementary study would be to investigate Nox2 in other cell types such as the cardiomyocyte or other inflammatory cells, therefore further dissecting cell specific roles in this disease setting such as pressure-overload or myocardial infarction. These studies would be able to assess whether the cell specific loss of Nox2 similarly reduces cardiac fibrosis and hypertrophy or whether the effects were particular to the setting of AngII-induced LVH and fibrosis. This would be important in determining the relevance of Nox2 in cardiac disease and in providing a better understanding.
- Ideally the mechanism to explain how Nox2 modulates macrophage migration is required to achieve a better understanding of the process. This will require the analysis of downstream signalling molecules to investigate where Nox2 is influencing the signalling process. Also further Dunn chamber chemotaxis experiments will be important to assess the effect of Nox2 in the migration of the macrophages towards different chemoattractants.
- TGF β downstream of AngII stimulation is important for the development of cardiac fibrosis and hypertrophy. TGF β demonstrated Nox2 dependent ROS production in fibroblasts. A more detailed investigation of the mechanism of action of TGF β by way of Nox2 dependent ROS production in fibroblasts

and cardiomyocytes, and relating this to fibroblast proliferation and transformation to myofibroblasts, will help in determining the relevance of Nox2 and providing a better understanding of the process.

6.6 FINAL CONCLUSIONS

In summary, the work undertaken in this thesis shows Nox2 in resident cardiac cells and in circulating peripheral inflammatory cells to have contrasting effects in AngII induced cardiac fibrosis and hypertrophy. The work showed Nox2 in macrophages to be important in their cellular migration, inflammatory phenotype and L-selectin expression, playing an important role in the development of cardiac hypertrophy. Whereas Nox2 in resident cardiac cells was crucial for the development of cardiac fibrosis wherein Nox2 dependent TGF β signalling in resident adult cardiac fibroblasts, at least in part, could be important. The findings demonstrate the widely varied effects of Nox2 modulation following AngII stimulation in different cell types. It suggests to separate mechanisms for the development of cardiac fibrosis and hypertrophy and thus offers the potential for their selective therapeutic management in the future.

7 PRESENTATIONS & PUBLICATIONS

7.1 PRESENTATIONS

- **S Chaubey**, MK Curtis, AM Shah, AC Cave. “Nox 2 NADPH oxidase-dependent ROS production in primary cardiac fibroblasts in response to angiotensin II and aldosterone.”
Poster presentation at The British Society of Cardiovascular Research (BSCR) scientific conference, Manchester. June 2008.
- **S Chaubey**, C Wells, G Jones, AM Shah, AC Cave. “NOX2-deficient bone derived macrophages exhibit defects in cell spreading and migration.”
Poster presentation at International Society of Heart Research (ISHR) scientific conference. Nice. June 2009.
- **S Chaubey**, C Wells, G Jones, AM Shah, AC Cave. “NOX2-deficient bone derived macrophages exhibit defects in cell spreading and migration.”
Oral presentation at The British Society of Cardiovascular Research (BSCR) scientific conference, Oxford. September 2009.
- **S Chaubey**, CE Murdoch, A Ivetic, B Yu, D Vanhoutte, S Heymans, A Brewer, AM Shah. “Cell-specific role of Nox2 NADPH oxidase in development of angiotensin II-induced cardiac fibrosis in vivo.”

Poster presentation at The British Cardiovascular Society (BCS) scientific conference. Manchester. June 2010.

7.2 PUBLICATIONS

- **S Chaubey**, AJ Ridley, CM Wells. (2011). Using the Dunn chemotaxis chamber to analyze primary cell migration in real time. Methods Mol Biol 769, 41-51.
- **S Chaubey**, G Jones, AM Shah, AC Cave, CM Wells (2013) “Nox2 is required for macrophage chemotaxis towards CSF-1”. PLoS One 8(2): e54869.
- CE Murdoch, **S Chaubey**, L Zeng, B Yu, A Ivetic, D Vanhoutte, S Heymans, DJ Grieve, AC Cave , AC Brewer , M Zhang , AM Shah. “Endothelial NADPH oxidase-2 promotes interstitial cardiac fibrosis and diastolic dysfunction through pro-inflammatory effects and endothelial-mesenchymal transition”.
Accepted in JACC

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